

In Re Ashkenazi
Application No. 09/993,234
Amended Appeal Brief



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application No.:	09/993,234	Confirmation No.:	1337
Applicant:	Avi J. ASHKENAZI		
Filed:	November 19, 2001		
Group Art Unit:	1642		
Examiner:	Gary B. Nickol		
Docket No.:	22338-01207		
Customer No.:	33,694		

AMENDED APPEAL BRIEF

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Washington, D.C. 20231

Dear Sir:

This Amended Appeal Brief is being filed in response to the Notification of Non-Compliant Appeal Brief dated March 24, 2006. While the Applicant believes that the original Appeal Brief was in full compliance with 37 CFR § 41.37, the headings in the brief and the table of contents have been amended as required in the Notification. The Amended Appeal Brief is being submitted within the one-month response time set out in the Notification and is therefore timely filed.

1. REAL PARTY IN INTEREST

The real party in interest in this appeal is Genentech, Inc. by virtue of an assignment recorded in the U.S. Patent and Trademark Office on September 2, 1997 at Reel/Frame: 008690/0298, in connection with parent U.S. Application No. 08/828,683, now U.S. Patent No. 6,469,144.

2. RELATED APPEALS AND INTERFERENCES

There are no other Appeals or Interferences known to the appellants, the appellants' legal representative, or assignee which will directly affect or be directly affected by or have a bearing on the Board's decision in the present pending appeal.

3. STATUS OF CLAIMS

Claims 1-45 were originally filed. Claims 46-94 were added and original claims 1-33 and 40-45 were cancelled at the time of filing in a preliminary amendment. Claims 35 and 46-94 were withdrawn by virtue of a Restriction Requirement maintained by the Examiner. Claims 34 and 36-39 were rejected in an Office Action dated October 7, 2003. Claims 34 and 36-39 were finally rejected in an Office Action dated December 14, 2004. The claims involved in this appeal, claims 34 and 36-39, are presented in the claim appendix attached hereto.

4. STATUS OF AMENDMENTS

There are no outstanding amendments.

5. SUMMARY OF CLAIMED SUBJECT MATTER

In one aspect, the present invention relates to an isolated nucleic acid encoding Apo-3 polypeptide comprising amino acid residues 1 to 417, 25 to 417, 25 to 198, or 338 to 417 of SEQ ID NO: 6, or a biologically active variant thereof. *See, e.g.*, Sequence ID NO:6, Figure and

Example 4 and specification pages 13, line 9 to page 14, line 14, page 20, lines 8-11, and page 23, lines 6-31. In another aspect, the present invention also relates to a vectors and host cells comprising the isolated nucleic acid. *See, e.g.*, page 22, line 31 to page 35, line 27, and example 4 and 5. In another aspect, the present invention also relates to a process of producing Apo-3 polypeptide. *See, e.g.*, page 22, line 31 to page 37, line 17 and example 4 and 5.

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

- A. Whether the specification provides written description support for claim 34 under 35 U.S.C. § 112, first paragraph.
- B. Whether claims 34 and 36-39 are anticipated, under 35 U.S.C. § 102(e), by U.S. Patent No. 6,153,402 (“Yu *et al.*”).

7. ARGUMENT

A. Claim 34 is supported by the disclosure in compliance with 35 U.S.C. § 112, first paragraph

Pending claim 34 was rejected under 35 U.S.C. § 112, first paragraph, as not supported by the specification. The Examiner has stated that “an isolated nucleic acid encoding Apo-3 polypeptide comprising amino acid residues 25-417 has no clear support in the specification and the claims as originally filed.” December 14, 2004 Office Action, page 4.

Amino acid residues 1-24 of SEQ ID NO:6 comprise a signal sequence. *See, e.g.*, the specification at page 20, lines 8-9; Figure 4. As described in the specification, amino acid residues 25-417 of SEQ ID NO:6 comprise the extracellular domain, transmembrane domain and intracellular domain of the Apo-3 polypeptide. *See, e.g.*, the specification at page 20, lines 9-11. Secreted forms of the Apo-3 polypeptide are contemplated and encompassed by the present claims. *See, e.g.*, the specification at page 13, lines 20-24. Cleavage of the Apo-3 signal sequence during secretion of the soluble protein yields a polypeptide having amino acid residues 25-417 of SEQ ID NO:6. Moreover, the specification at page 23, line 6-31, discusses certain

aspects of the signal sequence component. Signal sequences incorporating a specific cleavage site at the N-terminus of the mature protein, which are subsequently cleaved (yielding a polypeptide having amino acid residues 25-417 of SEQ ID NO:6) are described in this section. *See, e.g.*, the specification at page 23, lines 13-15. In any event, the present disclosure contemplates biologically active Apo-3 polypeptides having the amino acid sequence of SEQ ID NO:6, wherein “from about one to 24 amino acid residues are deleted.” *See, e.g.*, specification page 14, lines 1-4. This would include a polypeptide having amino acid residues 25-417 of SEQ ID NO:6.

Accordingly, an isolated nucleic acid encoding Apo-3 polypeptide comprising amino acid residues 25-417 of SEQ ID NO:6 is adequately described in the present specification.

B. Claims 34 and 36-39 are not anticipated by Yu *et al.* under 35 U.S.C. § 102(e)

Pending claims 34 and 36-39 were rejected under 35 U.S.C. § 102(e) as anticipated by Yu *et al.* The Examiner has stated that “applicants have not provided any clear evidence that the disclosure of DR3 or DR3-V1 does not anticipate the currently claimed subject matter.” In addition, the Examiner has stated that “Yu *et al.* clearly taught an isolated nucleic acid encoding amino acid residues 25-198 . . . [set forth] on page 67 of US Provisional 60/013,285 beginning at amino acid position No. 36.”

It has been the Appellant’s position that Yu *et al.* is not an appropriate reference under 35 U.S.C. § 102(e) because the priority application relied on for this citation, U.S. Provisional Application No. 60/013,285, fails to teach all of the elements, and indeed the isolated nucleic acid encoding Apo-3 polypeptide, of pending claim 34.

For purposes of the present argument, the Appellant notes that the Examiner has accorded the pending claims a priority date of September 23, 1996.¹ Yu *et al.* claims priority through three different provisional applications — U.S. Provisional Application No. 60/037,341, filed February 6, 1997; U.S. Provisional Application No. 60/028,711, filed October 17, 1996; and U.S. Provisional Application No. 60/013,285, filed March 12, 1996 (“the ’285 application”). Only the earliest of these provisional applications, the ’285 application, pre-dates the priority date currently accorded the pending claims. Therefore, the only relevant disclosure for determining whether Yu *et al.* anticipates under 35 U.S.C. § 102(e) is that of the ’285 application. *See, e.g., In re Wertheim and Mishkin*, 209 USPQ 554 (CCPA 1981).

Yu *et al.* discloses two different polypeptides, referred to as DR3-V1 and DR3 respectively, encoded by cDNA nucleic acid sequences which were cloned from a cDNA library. The ’285 application, however, disclosed *only* the sequence of DR3-V1²; the second sequence, DR3, was disclosed *for the first time* in the second priority application of Yu *et al.* filed October 17, 1996 and cannot, therefore, be prior art to the instant application. The fact that only DR3-V1 was disclosed in the earliest provisional application is significant because the DR3-V1 polypeptide, as disclosed in the ’285 application, does not correspond to the Apo-3 polypeptide in overall sequence or the particular regions identified in the present claims. This is clearly evident by the alignment of the signal peptides of the DR3-V1 sequence disclosed in the ’285 application and Apo-3 polypeptides below (bolded amino acid residues indicate identical residues).

DR3-V1:	1	Met	Glu	Glu	Thr	Gln	Gln	Gly	Glu	Ala	Pro	10
Apo-3:	1	Met	Glu	Gln	Arg	Pro	Arg	Gly	Cys	Ala	Ala	10
DR3-V1:	11	Arg	Gly	Gln	Leu	Arg	Gly	Glu	Ser	Ala	Ala	20
Apo-3:	11	Val	Ala	Ala	Ala	Leu	Leu	Leu	Val	Leu	Leu	20

¹ In order to minimize the issues on appeal Appellant accepts this priority date only for purposes of the present argument. The earliest claimed priority date of the present application is April 1, 1996. The Appellant expressly reserves the right to dispute the priority accorded to the present claims by the Examiner and to establish earlier priority than accorded the present claims.

² The ’285 application refers to DR3-V1 as “DDCR.” *See* Yu *et al.* Col. 3, ln. 22.

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DR3-V1:	21	Pro Val Pro Gln Ala Leu Leu Leu Val Leu	30
Apo-3:	21	Gly Ala Arg Ala	24

As is clearly evident, the signal peptides of DR3-V1 and Apo-3 are very different when aligned from the first amino acid residue of each polypeptide. The Appellant notes that there is no indication in the '285 application that the deduced DR3-V1 polypeptide should be compared with other proteins, if at all, in any other way than from the first amino acid residue.

An alignment of the designated extracellular domains of the DR3-V1 and Apo-3 polypeptides is as follows:³

DR3-V1:	30	Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr	39
Apo-3:	25	Gly Gly Thr Arg Ser Pro Arg Cys Asp Cys	34
DR3-V1:	40	Arg Ser Pro Arg Cys Asp Cys Ala Gly Asp	49
Apo-3:	35	Ala Gly Asp Phe His Lys Lys Ile Gly Leu	44
DR3-V1:	50	Phe His Lys Lys Ile Gly Leu Phe Cys Cys	59
Apo-3:	45	Phe Cys Cys Arg Gly Cys Pro Ala Gly His	54
DR3-V1:	60	Arg Gly Cys Pro Ala Gly His Tyr Leu Lys	69
Apo-3:	55	Tyr Leu Lys Ala Pro Cys Thr Glu Pro Cys	64
DR3-V1:	70	Ala Pro Cys Thr Glu Pro Cys Gly Asn Ser	79
Apo-3:	65	Gly Asn Ser Thr Cys Leu Val Cys Pro Gln	74
DR3-V1:	80	Thr Cys Leu Val Cys Pro Gln Asp Thr Phe	89
Apo-3:	75	Asp Thr Phe Leu Ala Trp Glu Asn His His	84
DR3-V1:	90	Leu Ala Trp Glu Asn His His Asn Ser Glu	99
Apo-3:	85	Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys	94
DR3-V1:	100	Cys Ala Arg Cys Gln Ala Cys Asp Glu Gln	109
Apo-3:	95	Asp Glu Gln Ala Ser Gln Val Ala Leu Glu	104
DR3-V1:	110	Ala Ser Gln Val Ala Leu Glu Asn Cys Ser	119
Apo-3:	105	Asn Cys Ser Ala Val Ala Asp Thr Arg Cys	114

³ This region is referred to as the "ligand binding domain" in the '285 application.

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DR3-V1:	120	Ala Val Ala Asp Thr Arg Cys Gly Cys Lys	129
Apo-3:	115	Gly Cys Lys Pro Gly Trp Phe Val Glu Cys	124
DR3-V1:	130	Pro Gly Trp Phe Val Glu Cys Gln Val Ser	139
Apo-3:	125	Gln Val Ser Gln Cys Val Ser Ser Ser Pro	134
DR3-V1:	140	Gln Cys Val Ser Ser Ser Pro Phe Tyr Cys	149
Apo-3:	135	Phe Tyr Cys Gln Pro Cys Leu Asp Cys Gly	144
DR3-V1:	150	Gln Pro Cys Leu Asp Cys Gly Ala Leu His	159
Apo-3:	145	Ala Leu His Arg His Thr Arg Leu Leu Cys	154
DR3-V1:	160	Arg His Thr Arg Leu Leu Cys Ser Arg Arg	169
Apo-3:	155	Ser Arg Arg Asp Thr Asp Cys Gly Thr Cys	164
DR3-V1:	170	Asp Thr Asp Cys Gly Thr Cys Leu Pro Gly	179
Apo-3:	165	Leu Pro Gly Phe Tyr Glu His Gly Asp Gly	174
DR3-V1:	180	Phe Tyr Glu His Gly Asp Gly Cys Val Ser	189
Apo-3:	175	Cys Val Ser Cys Pro Thr Ser Thr Leu Gly	184
DR3-V1:	190	Cys Pro Thr Ser Thr Leu Gly Ser Cys Pro	199
Apo-3:	185	Ser Cys Pro Glu Arg Cys Ala Ala Val Cys	194
DR3-V1:	200	Glu Arg Cys Ala Ala Val Cys Gly Trp Arg	209
Apo-3:	195	Gly Trp Arg Gln	198
DR3-V1:	210	Gln Met Phe Trp Val	215

As is also evident from the above alignment, the extracellular domains of DR3-V1 and Apo-3 are very different.

Finally, an alignment of the designated death domains of the DR3-V1 and Apo-3 polypeptides is as follows:

DR3-V1:	350	Met Asp Ala Val Pro Ala Arg Arg Trp Lys	359
Apo-3:	338	Val Met Asp Ala Val Pro Ala Arg Arg Trp	347
DR3-V1:	360	Glu Phe Val Arg Thr Leu Gly Leu Arg Glu	369
Apo-3:	348	Lys Glu Phe Val Arg Thr Leu Gly Leu Arg	357

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DR3-V1:	370	Ala	Glu	Ile	Glu	Ala	Val	Glu	Val	Glu	Ile	379
Apo-3:	358	Glu	Ala	Glu	Ile	Glu	Ala	Val	Glu	Val	Glu	367
DR3-V1:	380	Gly	Arg	Phe	Arg	Asp	Gln	Gln	Tyr	Glu	Met	389
Apo-3:	368	Ile	Gly	Arg	Phe	Arg	Asp	Gln	Gln	Tyr	Glu	377
DR3-V1:	390	Leu	Lys	Arg	Trp	Arg	Gln	Gln	Gln	Pro	Ala	399
Apo-3:	378	Met	Leu	Lys	Arg	Trp	Arg	Gln	Gln	Gln	Pro	387
DR3-V1:	400	Gly	Leu	Gly	Ala	Val	Tyr	Ala	Ala	Leu	Glu	409
Apo-3:	388	Ala	Gly	Leu	Gly	Ala	Val	Tyr	Ala	Ala	Leu	397
DR3-V1:	410	Arg	Met	Gly	Leu	Asp	Gly	Cys	Val	Glu	Asp	419
Apo-3:	398	Glu	Arg	Met	Gly	Leu	Asp	Gly	Cys	Val	Glu	407
DR3-V1:	420	Leu										
Apo-3:	408	Asp	Leu	Arg	Ser	Arg	Leu	Gln	Arg	Gly	Pro	417

As is similarly evident from the above alignment, the death domains of DR3-V1 and Apo-3 are markedly different.

To the extent the '285 application contemplates fragments of the deduced DR3-V1 polypeptide, no fragments having any likeness to the Apo-3 polypeptide of the present claims are identified in the '285 application. Moreover, the '285 application fails to discuss any specific sequence frame shifts, inversions, repeats, additions or deletions that would result in a polypeptide having any likeness to the Apo-3 polypeptide of the present claims. It is possible that the Examiner may have relied on such alterations in the deduced DR3-V1 polypeptide sequence but has not identified where in the '285 application one may locate the basis for the suggested alterations nor indicated that they are inherent aspects of the deduced DR3-V1 polypeptide. For example, although the Examiner has noted a comparison of a segment of the deduced DR3-V1 polypeptide starting at amino acid position No. 36 with a portion of the Apo-3 polypeptide of the present claims, this alignment appears to be impermissibly based either on the knowledge of the sequence of the present claims or the disclosure from Yu *et al.* (see, e.g., Col. 5, line 63) that was not included in the '285 application. Such an alignment of the sequences beginning at amino acid position No. 36 does not involve any region of interest identified in the

'285 application. The '285 application provides no indication that a region of the deduced DR3-V1 polypeptide starting at amino acid position No. 36 exists as a separate polypeptide, begins a region of interest in the DR3-V1 polypeptide (*i.e.*, extracellular domain), or has any significance whatsoever.

Accordingly, the above alignments provide evidence supporting the Appellant's position that the deduced DR3-V1 polypeptide does not correspond to, and therefore does not anticipate under § 102(e), the Apo-3 polypeptide of the present claims.

In addition, to the extent the '285 application provides any direction to a signal peptide (amino acid residues 1-30 – *see* page 7, lines 34-35), extracellular domain (amino acid residues 30-215 - *see* page 7, line 35), transmembrane domain (amino acid residues 215-240 – *see* page 8, line 1), intracellular domain (amino acid residues 240-428 – *see* page 8, lines 1-2) or death domain (amino acid residues 350-420 – *see* page 8, lines 2-3), it points to completely different domains having different boundaries within its longer 428 amino acid DR3-V1 (DDCR) polypeptide versus the polypeptide of the present claims.⁴ *See* '285 application at page 7, line 34-page 8, line 3; page 11, lines 10-19; Figure 1; SEQ ID NO:1 as is illustrated in the sequence alignments above.

In this regard, the Appellant notes that the domains (*e.g.*, the signal sequence,

⁴ *See In re Arkley*, 172 USPQ 524 (CCPA 1972) (“[F]or the instant rejection under 35 U.S.C. 102(e) to have been proper, the . . . reference must clearly and unequivocally disclose the claimed compound or direct those skilled in the art to the compound without any need for picking, choosing, and combining various disclosures not directly related to each other by the teachings of the cited reference.”); *see also Suntiger, Inc. v. Blublocker*, 51 USPQ2d 1811, 1818 (Fed. Cir. 1999) (“The case law makes clear that disclosure of a generic expression encompassing a large number of possible variants is not a description of all of them.” *Id.* (citing *In re Ruschig*, 154 USPQ 118, 121 (CCPA 1967) (finding that a disclosure of a class of compounds did not provide descriptive support for a single compound within the class *not specifically identified* in the specification); and *Fujikawa v. Wattanasin*, 39 USPQ2d 1895, 1904-05 (Fed. Cir. 1996) (finding no descriptive support even when the proposed genus was actually disclosed in the specification due to the *lack of direction* that would lead one of skill to the proposed subgenus)).

extracellular domain, transmembrane domain, intracellular domain and death domain) of DR3-V1 set forth in Yu *et al.* are not those set forth for DR3-V1 in the '285 application. Compare the '285 application at page 7, line 34-page 8, line 3; page 11, lines 10-19 with Yu *et al.* at col. 4, lines 25-31. The table below summarizes the changes to these domains between the '285 application and Yu *et al.*:

DR3-V1 Domain	'285 application (SEQ ID NO:2) Amino Acid Position Nos.	Yu <i>et al.</i> (SEQ ID NO:2) Amino Acid Position Nos.
Signal peptide	1-30	1-35
Extracellular domain	30-215	36-212
Transmembrane domain	215-240	213-235
Intracellular domain	240-428	236-428
Death domain	350-420	353-419

Each of these domains were changed, without explanation, in applications filed after the '285 application. *See id.*; *see also* Application No. 60/028,711 at page 6, lines 15-19. One likely explanation for these changes is that sometime between the filing of the '285 and the application giving rise to Yu *et al.*, the inventors of the '285 application realized that they inaccurately estimated the positions of each of the DR3-V1 polypeptide domains in the '285 application. In fact, Yu *et al.* acknowledges that the position of the signal sequence cleavage site, for example, may have been predicted using a method having only about 75-80% accuracy. *See* Yu *et al.* at col. 6, lines 50-59 (a disclosure that was provided in Yu *et al.*, but not in the '285 application). Clearly, a question of descriptive support for, and enablement of, any DR3-V1 polypeptide that may exist in nature arises in light of the comparison of the '285 application and Yu *et al.* disclosures. Moreover, confusion by the Yu *et al.* and '285 application inventors about aspects of the deduced DR3-V1 polypeptide cannot be reasonably interpreted as an inherent disclosure of the Apo-3 polypeptide of the present claims, with each of its limitations. *See Continental Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 1269, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) ("Inherency, however, may not be established by probabilities or possibilities.").

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Accordingly, the Yu *et al.* reference is not an effective anticipatory reference under 35 U.S.C. § 102(e) as its priority applications do not describe, in a manner sufficient under § 112, each of the limitations of the pending claims.

Conclusion

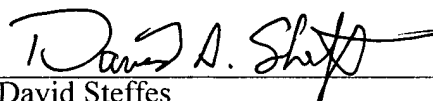
Appellants have addressed each of the rejections set forth by the Examiner. For the reasons stated above, it is respectfully submitted that the final rejections of claims 34 and 36-39 under 35 U.S.C. § 112, first paragraph, and § 102(e) are in error and warrant reversal of the rejections by the Board.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, appellant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 18-1260** referencing docket no. 2233801207. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: April 21, 2006

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8. CLAIM APPENDIX

34. (Previously presented) Isolated nucleic acid encoding Apo-3 polypeptide comprising amino acid residues 1 to 417, 25 to 417, 25 to 198, or 338 to 417 of SEQ ID NO: 6, or a biologically active variant thereof.

36. (Original) A vector comprising the nucleic acid of claim 34.

37. (Original) The vector of claim 36 operably linked to control sequences recognized by a host cell transformed with the vector.

38. (Original) A host cell comprising the vector if claim 36.

39. (Previously presented) A process of producing Apo-3 polypeptide comprising culturing the host cell of claim 38 and isolating said polypeptide.

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9. EVIDENCE APPENDIX

- Specification and Claims of U.S. Provisional Application No. 60/013,285; and
- Specification and Claims of U.S. Provisional Application No. 60/028,711 are attached hereto.

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	TITLE DEATH DOMAIN CONTAINING RECEPTOR				
This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above. By authority of the COMMISSIONER OF PATENTS AND TRADEMARKS NOV 5 1996 Certifying Officer <i>Mittie E. [Signature]</i>					

PATENT APPLICATION SERIAL NO. **60/013285**

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N: 141WGS507

DEATH DOMAIN CONTAINING RECEPTOR

The present invention relates to a novel member of the tumor necrosis factor family of receptors.

BACKGROUND OF THE INVENTION

Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intra-cellular response.

For example, tumor necrosis factors (TNF)alpha and beta are cytokines which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

Among the ligands there are included TNF- α , lymphotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40L, CD27L, CD30L, 4-1BBL, OX40L and nerve growth factor (NGF). The superfamily of TNF

receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 4-1BB, X40, low affinity p75 and NGF-receptor (Meager, A., *Biologicals*, 22:291-295 (1994)).

Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., *supra*).

Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., et al., *Nature*, 356:314 (1992)), perhaps reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (Allen, R.C., et al., *Science*, 259:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by faulty sensory innervation of peripheral structures (Lee, K.F., et al., *Cell*, 69:737 (1992)).

TNF and LT- α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT- α , acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT- α are involved in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von

1 Huffel, C., Science, 264:667, 668 (1994). Mutations in the
2 p55 Receptor cause increased susceptibility to microbial
3 infection.

4 Moreover, an about 80 amino acid domain near the C-
5 terminus of TNFR1 (p55) and Fas was reported as the "death
6 domain," which is responsible for transducing signals for
7 programmed cell death (Tartaglia et al., Cell 74:845 (1993)).

8 The effects of TNF family ligands and TNF family
9 receptors are varied and influence numerous functions, both
10 normal and abnormal, in the biological processes of the
11 mammalian system. There is a clear need, therefore, for
12 identification and characterization of such receptors and
13 ligands that influence biological activity, both normally and
14 in disease states. In particular, there is a need to isolate
15 and characterize novel members of the TNF receptor family.

16 SUMMARY OF THE INVENTION

17
18 The present inventors have discovered a novel member of
19 the tumor necrosis factor (TNF) family of receptors. By the
20 invention, isolated nucleic acid molecules are provided
21 comprising nucleic acid sequences encoding a death domain
22 containing receptor (DDCR), and fragments thereof. The DDCR
23 gene contains an open reading frame encoding a protein of
24 about 428 amino acid residues whose initiation codon is at
25 position 198-200 of the nucleotide sequence shown in Figure
26 1 [SEQ ID NO. 1], with a leader sequence of about 30 amino
27 acid residues, and a deduced molecular weight of about 47
28 kDa. Of known members of the TNF receptor family, the DDCR
29 polypeptide shares the greatest degree of homology with human
30 TNF R1.
31

32 Isolated nucleic acid molecules of the present invention
33 include DNA molecules comprising an open reading frame (ORF)
34 whose initiation codon is at position 198-200 of the
35 nucleotide sequence shown in Figure 1 [SEQ ID NO 1] and

1 further include DNA molecules which comprise a sequence
2 substantially different than all or part of the ORF whose
3 initiation codon is at position 198-200 of the nucleotide
4 sequence shown in Figure 1 (SEQ ID NO 1) but which, due to
5 the degeneracy of the genetic code, still encode the DDCR
6 polypeptide or a fragment thereof.

7 Preferred nucleic acid fragments of the present
8 invention include nucleic acid molecules encoding: a
9 polypeptide comprising the DDCR ligand binding domain; a
10 polypeptide comprising the DDCR transmembrane domain; a
11 polypeptide comprising the DDCR intracellular domain; and a
12 polypeptide comprising the DDCR death domain.

13 The present invention further relates to variants of the
14 nucleic acid molecules of the present invention, which encode
15 for fragments, analogs or derivatives of the DDCR
16 polypeptide. Variants may occur naturally, such as an allelic
17 variant. Non-naturally occurring variants may be produced
18 using art-known mutagenesis techniques.

19 Further embodiments of the invention include isolated
20 nucleic acid molecules that are at least 70% identical, and
21 more preferably at least 80%, 90%, 95%, 97%, 98% or 99%
22 identical, to the nucleic acid sequence shown in Figure 1
23 (SEQ ID NO 1), to fragments complementary thereto, or to
24 fragments thereof selected from (1) a fragment that encodes
25 the mature DDCR protein (i.e., the open reading frame), (2)
26 a fragment that encodes the DDCR ligand binding domain, (3)
27 a fragment that encodes the DDCR transmembrane domain, (4) a
28 fragment that encodes the DDCR intracellular domain, and (5)
29 a fragment that encodes the death domain.

30 The invention is also related to the use of the DDCR
31 polynucleotides for detecting altered expression of DDCR
32 transcripts, such as, for example, during apoptosis
33 dysfunction associated with tumors or autoimmune disease.

34 The isolated nucleic acid molecules of the present
35 invention are also valuable for chromosome identification.

1 The DDCR nucleotide sequence is specifically targeted to and
2 can hybridize with a particular location on an individual
3 human chromosome.

4 The present invention also provides vectors and host
5 cells for recombinant expression of the nucleic acid
6 molecules described herein.

7 Further provided are isolated DDCR polypeptides having
8 the amino acid sequence shown in Figure 1 (SEQ ID NO:2), or
9 fragments thereof. The polypeptides of the present invention
10 are preferably provided in an isolated form, and preferably
11 are substantially purified. A recombinantly produced version
12 of the DDCR polypeptide is substantially purified by the one-
13 step method described in Smith and Johnson, *Gene* 67:31-40
14 (1988).

15 The polypeptides of the present invention include the
16 polypeptide of SEQ ID NO:2 (in particular the mature
17 polypeptide) as well as polypeptides which have at least 70%
18 similarity (preferably at least 70% identity), to the
19 polypeptide of SEQ ID NO:2 and more preferably at least 90%
20 similarity (more preferably at least 90% identity) to the
21 polypeptide of SEQ ID NO:2 and still more preferably at least
22 95% similarity (still more preferably at least 95% identity)
23 to the polypeptide of SEQ ID NO:2 and also include portions
24 of such polypeptides with such portion of the polypeptide
25 generally containing at least 30 amino acids and more
26 preferably at least 50 amino acids.

27 The invention further provides DDCR polypeptide
28 fragments selected from the mature DDCR protein, the DDCR
29 ligand binding domain, the DDCR transmembrane domain, the
30 DDCR intracellular domain, and the DDCR death domain. Such
31 fragments of DDCR can be used to raise polyclonal and
32 monoclonal antibody-agonists and antagonists capable of
33 enhancing or inhibiting the DDCR ligand/receptor signaling
34 pathway. Further, such fragments can be used in the yeast
35 two-hybrid system to "capture" DDCR binding proteins which are

1 also candidate agonist and antagonist according to the
2 present invention.

3 The present invention also relates to diagnostic assays
4 such as quantitative and diagnostic assays for detecting
5 levels of DDCR protein, or the soluble form thereof, in cells
6 and tissues, including determination of normal and abnormal
7 levels. Thus, for instance, a diagnostic assay in accordance
8 with the invention for detecting over-expression of DDCR, or
9 soluble form thereof, compared to normal control tissue
10 samples may be used to detect the presence of tumors.

11 Tumor Necrosis Factor (TNF) family ligands are known to be
12 among the most pleiotropic cytokines, inducing a large number
13 of cellular responses, including cytotoxicity, anti-viral
14 activity, immunoregulatory activities, and the
15 transcriptional regulation of several genes. Cellular
16 response to TNF-family ligands include not only normal
17 physiological responses, but also diseases associated with
18 increased apoptosis or the inhibition of apoptosis.
19 Apoptosis-programmed cell death-is a physiological mechanism
20 involved in the deletion of peripheral T lymphocytes of the
21 immune system, and its dysregulation can lead to a number of
22 different pathogenic processes. Diseases associated with
23 increased cell survival, or the inhibition of apoptosis,
24 include cancers, autoimmune disorders, viral infections,
25 inflammation, graft v. host disease, acute graft rejection,
26 and chronic graft rejection. Diseases associated with
27 increased apoptosis include AIDS, neurodegenerative
28 disorders, myelodysplastic syndromes, ischemic injury, toxin-
29 induced liver disease, septic shock, cachexia and anorexia.

30 Thus, the invention further provides a method for
31 enhancing apoptosis induced by a TNF-family ligand, which
32 involves administering to a cell which expresses the DDCR
33 polypeptide an effective amount of an agonist capable of
34 increasing DDCR mediated signaling. Preferably, DDCR
35 mediated signaling is increased to treat a disease wherein

1 decreased apoptosis is exhibited.

2 In a further aspect, the present invention is directed
3 to a method for inhibiting apoptosis induced by a TNF-family
4 ligand, which involves administering to a cell which
5 expresses the DDCR polypeptide an effective amount of an
6 antagonist capable of decreasing DDCR mediated signaling.
7 Preferably, DDCR mediated signaling is decreased to treat a
8 disease wherein increased apoptosis is exhibited.

9 Whether any candidate "agonist" or "antagonist" of the
10 present invention can enhance or inhibit apoptosis can be
11 determined using art-known TNF-family ligand/receptor
12 cellular response assays, including those described in more
13 detail below. Thus, in a further aspect, a screening method
14 is provided for determining whether a candidate agonist or
15 antagonist is capable of enhancing or inhibiting a cellular
16 response to a TNF-family ligand. The method involves
17 contacting cells which express the DDCR polypeptide with a
18 candidate compound and a TNF-family ligand, assaying a
19 cellular response, and comparing the cellular response to a
20 standard cellular response, the standard being assayed when
21 contact is made with the ligand in absence of the candidate
22 compound, whereby an increased cellular response over the
23 standard indicates that the candidate compound is an agonist
24 of the ligand/receptor signaling pathway and a decreased
25 cellular response compared to the standard indicates that the
26 candidate compound is an antagonist of the ligand/receptor
27 signaling pathway. By the invention, a cell expressing the
28 DDCR polypeptide can be contacted with either an endogenous
29 or exogenously administered TNF-family ligand.

30 BRIEF DESCRIPTION OF THE DRAWINGS

31
32
33 Figure 1 shows the nucleotide and deduced amino acid
34 sequence of DDCR. Amino acids 1 to 30 constitute the signal
35 peptide, amino acids 30-215 the ligand binding domain, amino

1 acids 215-240 the transmembrane domain, amino acids 240-428
2 the intracellular domain, and amino acids 350-420 the death
3 domain.

4 Figure 2 shows the regions of similarity between the
5 amino acid sequences of the DDCR (referred to in the Figure
6 as HTTNB61.pap), human tumor necrosis factor receptor 1, and
7 Fas receptor (SEQ ID NOs:3 and 4).

8 Figure 3 shows structural and functional features of
9 DDCR deduced by the indicated techniques, as a function of
10 amino acid sequence.

12 DETAILED DESCRIPTION OF THE INVENTION

13
14 The present invention provides isolated nucleic acid
15 molecules comprising a nucleic acid sequence encoding the
16 DDCR polypeptide whose amino acid sequence is shown in Figure
17 1 [SEQ ID NO:2] or a fragment of the polypeptide. The DDCR
18 polypeptide of the present invention shares sequence homology
19 with human TNF RI and Fas (Figure 3). The nucleotide
20 sequence shown in Figure 1 [SEQ ID NO:1] was obtained by
21 sequencing the HTTNB61 clone, which was deposited on February
22 29, 1996 at the American Type Culture Collection, 12301 Park
23 Lawn Drive, Rockville, Maryland 20852, and given accession
24 number 97456.

25 Thus, in one aspect, isolated nucleic acid molecules are
26 provided which encode the DDCR polypeptide. By "isolated"
27 nucleic acid molecule(s) is intended a nucleic acid molecule,
28 DNA or RNA, which has been removed from its native
29 environment. For example, recombinant DNA molecules
30 contained in a vector are considered isolated for the
31 purposes of the present invention. Further examples of
32 isolated DNA molecules include recombinant DNA molecules
33 maintained in heterologous host cells or purified (partially
34 or substantially) DNA molecules in solution. Isolated RNA
35 molecules include in vitro RNA transcripts of the DNA

1 molecules of the present invention. By "isolated" polypeptide
2 or protein is intended a polypeptide or protein removed from
3 its native environment. For example, recombinantly produced
4 polypeptides and proteins expressed in host cells are
5 considered isolated for purposes of the invention as are
6 native or recombinant polypeptides which have been
7 substantially purified by any suitable technique such as, for
8 example, the single-step purification method disclosed in
9 Smith and Johnson, *Gene* 67:31-40 (1988).

10 Using the information provided herein, such as the
11 nucleic acid sequence set out in Figure 1, a nucleic acid
12 molecule of the present invention encoding a DDCR polypeptide
13 may be obtained using standard cloning and screening
14 procedures, such as those for cloning cDNAs using mRNA as
15 starting material. Illustrative of the invention, the
16 nucleic acid molecule described in Figure 1 was discovered in
17 a cDNA library derived from cells of a human testis tumor.

18 The DDCR gene contains an open reading frame encoding a
19 protein of about 428 amino acid residues whose initiation
20 codon is at position 198-200 of the nucleotide sequence shown
21 in Figure 1 [SEQ ID NO. 1], with a leader sequence of about
22 30 amino acid residues, and a deduced molecular weight of
23 about 47 kDa. Of known members of the TNF receptor family,
24 the DDCR polypeptide of the invention shares the greatest
25 degree of homology with human TNF R1. (The DDCR polypeptide
26 shown in Figure 1 [SEQ ID NO. 2] is about 20% identical and
27 about 50% similar to human TNF R1.)

28 As indicated, nucleic acid molecules of the present
29 invention may be in the form of RNA, such as mRNA, or in the
30 form of DNA, including, for instance, cDNA and genomic DNA
31 obtained by cloning or produced synthetically. The DNA may
32 be double-stranded or single-stranded. Single-stranded DNA
33 may be the coding strand, also known as the sense strand, or
34 it may be the non-coding strand, also referred to as the
35 anti-sense strand.

1 Isolated nucleic acid molecules of the present invention
2 include DNA molecules comprising an open reading frame (ORF)
3 whose initiation codon is at position 198-200 of the
4 nucleotide sequence shown in Figure 1 [SEQ ID NO 1] and
5 further include DNA molecules which comprise a sequence
6 substantially different than all or part of the ORF whose
7 initiation codon is at position 198-200 of the nucleotide
8 sequence shown in Figure 1 [SEQ ID NO 1] but which, due to
9 the degeneracy of the genetic code, still encode the DDCR
10 polypeptide or a fragment thereof. Of course, the genetic
11 code is well known in the art. Thus, it would be routine for
12 one skilled in the art to generate the degenerate variants
13 described above.

14 The invention further provides an isolated nucleic acid
15 molecule having the nucleotide sequence shown in Figure 1
16 [SEQ ID NO 1], or a fragment thereof. Such isolated DNA
17 molecules and fragments thereof are useful as DNA probes for
18 gene mapping by *in situ* hybridization with chromosomes and
19 for detecting expression of the DDCR gene in human tissue
20 (including testis tumor tissue) by Northern blot analysis.
21 Of course, as discussed above, if a DNA molecule includes the
22 ORF whose initiation codon is at position 198-200 of Figure
23 1 [SEQ ID NO 1], then it is also useful for expressing the
24 DDCR polypeptide or a fragment thereof.

25 By fragments of an isolated DNA molecule having the
26 nucleotide sequence shown in Figure 1 [SEQ ID NO 1] are
27 intended DNA fragments at least 20 bp, and more preferably at
28 least 30 bp in length which are useful as DNA probes as
29 discussed above. Of course larger DNA fragments 50-1500 bp
30 in length are also useful as DNA probes according to the
31 present invention as are DNA fragments corresponding to most,
32 if not all, of the nucleotide sequence shown in Figure 1 [SEQ
33 ID NO 1]. By a fragment at least 20 bp in length, for
34 example, is intended fragments which include 20 or more bases
35 from the nucleotide sequence in Figure 1 [SEQ ID NO 1].

1 Since the nucleotide sequence shown in Figure 1 (SEQ ID
2 NO 1) is provided, generating such DNA fragments would be
3 routine to the skilled artisan. For example, restriction
4 endonuclease cleavage or shearing by sonication could easily
5 be used to generate fragments of various sizes.
6 Alternatively, the DNA fragments of the present invention
7 could be generated synthetically according to known
8 techniques.

9 Preferred nucleic acid fragments of the present
10 invention include nucleic acid molecules encoding: a
11 polypeptide comprising the DDCR ligand binding domain (amino
12 acid residues from about 30 to about 215 in Figure 1 (SEQ ID
13 NO 2)); a polypeptide comprising the DDCR transmembrane
14 domain (amino acid residues from about 215 to about 240 in
15 Figure 1 (SEQ ID NO 2)); a polypeptide comprising the DDCR
16 intracellular domain (amino acid residues from about 240 to
17 about 428 in Figure 1 (SEQ ID NO 2)); and a polypeptide
18 comprising the DDCR death domain (amino acid residues from
19 about 350 to about 420 in Figure 1 (SEQ ID NO 2)). It will
20 be appreciated that reasonable persons of skill in the art
21 may disagree, depending on the criteria used, concerning the
22 exact "address" of the above-described DDCR domains. Thus,
23 for example, the exact location of the DDCR ligand binding
24 domain in Figure 1 (SEQ ID NO 2) may vary slightly (e.g., the
25 address may "shift" by about 1 to 5 residues) depending on the
criteria used to define the domain.

27 As indicated, nucleic acid molecules of the present
28 invention which encode the DDCR polypeptide may include, but
29 are not limited to the coding sequence for the mature
30 polypeptide, by itself; the coding sequence for the mature
31 polypeptide and additional sequences, such as those encoding
32 a leader or secretory sequence, such as a pre-, or pro- or
33 prepro- protein sequence; the coding sequence of the mature
34 polypeptide, with or without the aforementioned additional
35 coding sequences, together with additional, non-coding

sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci., USA 86: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson et al., Cell 37: 767 (1984), for instance.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode for fragments, analogs or derivatives of the DCCR polypeptide. Variants may occur naturally, such as an allelic variant. Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

Especially preferred among these are silent substitutions, additions and deletions, which do not alter

the properties and activities of the DDCR polypeptide or fragment thereof. Also especially preferred in this regard are conservative substitutions. Most highly preferred are nucleic acid molecules encoding the DDCR polypeptide having the amino acid sequence of Figure 1 (SEQ ID NO 2).

Further embodiments of the invention include isolated nucleic acid molecules that are at least 70% identical, and more preferably at least 80%, 90%, 95%, 97%, 98% or 99% identical, to the nucleic acid sequence shown in Figure 1 (SEQ ID NO 1), to fragments complementary thereto, or to fragments thereof selected from (1) a fragment that encodes the mature DDCR protein (i.e., the open reading frame), (2) a fragment that encodes the DDCR ligand binding domain, (3) a fragment that encodes the DDCR transmembrane domain, (4) a fragment that encodes the DDCR intracellular domain, and (5) a fragment that encodes the death domain.

The invention is further related to nucleic acid molecules that hybridize under stringent conditions to the above-described nucleic acid molecules. By "stringent conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5xSSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 ug/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

Polynucleotide assays

This invention is also related to the use of the DDCR polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of DDCR associated with a dysfunction will

1 provide a diagnostic tool that can add or define a diagnosis
2 of a disease or susceptibility to a disease which results
3 from under-expression over-expression or altered expression
4 of DDCR or a soluble form thereof, such as, for example,
5 tumors or autoimmune disease.

6 Individuals carrying mutations in the DDCR gene may be
7 detected at the DNA level by a variety of techniques. Nucleic
8 acids for diagnosis may be obtained from a patient's cells,
9 such as from blood, urine, saliva, tissue biopsy and autopsy
10 material. The genomic DNA may be used directly for detection
11 or may be amplified enzymatically by using PCR prior to
12 analysis. (Saiki et al., Nature, 324: 163-166 (1986)). RNA
13 or cDNA may also be used in the same ways. As an example,
14 PCR primers complementary to the nucleic acid encoding DDCR
15 can be used to identify and analyze DDCR expression and
16 mutations. For example, deletions and insertions can be
17 detected by a change in size of the amplified product in
18 comparison to the normal genotype. Point mutations can be
19 identified by hybridizing amplified DNA to radiolabeled DDCR
20 RNA or alternatively, radiolabeled DDCR antisense DNA
21 sequences. Perfectly matched sequences can be distinguished
22 from mismatched duplexes by RNase A digestion or by
23 differences in melting temperatures.

24 Sequence differences between a reference gene and genes
25 having mutations also may be revealed by direct DNA
26 sequencing. In addition, cloned DNA segments may be employed
27 as probes to detect specific DNA segments. The sensitivity
28 of such methods can be greatly enhanced by appropriate use of
29 PCR or another amplification method. For example, a
30 sequencing primer is used with double-stranded PCR product or
31 a single-stranded template molecule generated by a modified
32 PCR. The sequence determination is performed by conventional
33 procedures with radiolabeled nucleotide or by automatic
34 sequencing procedures with fluorescent-tags.

1 Genetic testing based on DNA sequence differences may be
2 achieved by detection of alteration in electrophoretic
3 mobility of DNA fragments in gels, with or without denaturing
4 agents. Small sequence deletions and insertions can be
5 visualized by high resolution gel electrophoresis. DNA
6 fragments of different sequences may be distinguished on
7 denaturing formamide gradient gels in which the mobilities of
8 different DNA fragments are retarded in the gel at different
9 positions according to their specific melting or partial
10 melting temperatures (see, e.g., Myers et al., Science, 230:
11 1242 (1985)).

12 Sequence changes at specific locations also may be
13 revealed by nuclease protection assays, such as RNase and S1
14 protection or the chemical cleavage method (e.g., Cotton et
15 al., Proc. Natl. Acad. Sci., USA, 85: 4397-4401 (1985)).

16 Thus, the detection of a specific DNA sequence may be
17 achieved by methods such as hybridization, RNase protection,
18 chemical cleavage, direct DNA sequencing or the use of
19 restriction enzymes, (e.g., restriction fragment length
20 polymorphisms ("RFLP") and Southern blotting of genomic DNA.

21 In addition to more conventional gel-electrophoresis and
22 DNA sequencing, mutations also can be detected by in situ
23 analysis.

24 **Chromosome assays**

25 The sequences of the present invention are also valuable
26 for chromosome identification. The sequence is specifically
27 targeted to and can hybridize with a particular location on
28 an individual human chromosome. Moreover, there is a current
29 need for identifying particular sites on the chromosome. Few
30 chromosome marking reagents based on actual sequence data
31 (repeat polymorphisms) are presently available for marking
32 chromosomal location. The mapping of DNAs to chromosomes
33 according to the present invention is an important first step
34
35

1 in correlating those sequences with genes associated with
2 disease.

3 In certain preferred embodiments in this regard, the
4 cDNA herein disclosed is used to clone genomic DNA of a DDCR
5 gene. This can be accomplished using a variety of well known
6 techniques and libraries, which generally are available
7 commercially. The genomic DNA the is used for in situ
8 chromosome mapping using well known techniques for this
9 purpose. Typically, in accordance with routine procedures
10 for chromosome mapping, some trial and error may be necessary
11 to identify a genomic probe that gives a good in situ
12 hybridization signal.

13 In some cases, in addition, sequences can be mapped to
14 chromosomes by preparing PCR primers (preferably 15-25 bp)
15 from the cDNA. Computer analysis of the 3' untranslated
16 region of the gene is used to rapidly select primers that do
17 not span more than one exon in the genomic DNA, thus
18 complicating the amplification process. These primers are
19 then used for PCR screening of somatic cell hybrids
20 containing individual human chromosomes. Only those hybrids
21 containing the human gene corresponding to the primer will
22 yield an amplified fragment.

23 PCR mapping of somatic cell hybrids is a rapid procedure
24 for assigning a particular DNA to a particular chromosome.
25 Using the present invention with the same oligonucleotide
26 primers, sublocalization can be achieved with panels of
27 fragments from specific chromosomes or pools of large genomic
28 clones in an analogous manner. Other mapping strategies that
29 can similarly be used to map to its chromosome include in
30 situ hybridization, prescreening with labeled flow-sorted
31 chromosomes and preselection by hybridization to construct
32 chromosome specific-cDNA libraries.

33 Fluorescence in situ hybridization ("FISH") of a cDNA
34 clone to a metaphase chromosomal spread can be used to
35 provide a precise chromosomal location in one step. This

1 technique can be used with cDNA as short as 50 or 60. For a
2 review of this technique, see Verma et al., HUMAN
3 CHROMOSOMES: A MANUAL OF BASIC TECHNIQUES, Pergamon Press,
4 New York (1988).

5 Once a sequence has been mapped to a precise chromosomal
6 location, the physical position of the sequence on the
7 chromosome can be correlated with genetic map data. Such
8 data are found, for example, in V. McKusick, MENDELIAN
9 INHERITANCE IN MAN, available on line through Johns Hopkins
10 University, Welch Medical Library. The relationship between
11 genes and diseases that have been mapped to the same
12 chromosomal region are then identified through linkage
13 analysis (coinheritance of physically adjacent genes).

14 Next, it is necessary to determine the differences in
15 the cDNA or genomic sequence between affected and unaffected
16 individuals. If a mutation is observed in some or all of the
17 affected individuals but not in any normal individuals, then
18 the mutation is likely to be the causative agent of the
19 disease.

20 With current resolution of physical mapping and genetic
21 mapping techniques, a cDNA precisely localized to a
22 chromosomal region associated with the disease could be one
23 of between 50 and 500 potential causative genes. (This
24 assumes 1 megabase mapping resolution and one gene per 20
25 kb).

26 **Vectors and Host Cells**

27 The present invention also relates to vectors which
28 include DNA molecules of the present invention, host cells
29 which are genetically engineered with vectors of the
30 invention and the production of polypeptides of the invention
31 by recombinant techniques.

32 Host cells can be genetically engineered to incorporate
33 nucleic acid molecules and express polypeptides of the
34
35

1 present invention. For instance, nucleic acid molecules may
2 be introduced into host cells using well known techniques of
3 infection, transduction, transfection, transvection and
4 transformation. The polynucleotides may be introduced alone
5 or with other polynucleotides. Such other polynucleotides
6 may be introduced independently, co-introduced or introduced
7 joined to the polynucleotides of the invention.

8 Thus, for instance, polynucleotides of the invention may
9 be transfected into host cells with another, separate,
10 polynucleotide encoding a selectable marker, using standard
11 techniques for co-transfection and selection in, for
12 instance, mammalian cells. In this case the polynucleotides
13 generally will be stably incorporated into the host cell
14 genome.

15 Alternatively, the polynucleotides may be joined to a
16 vector containing a selectable marker for propagation in a
17 host. The vector construct may be introduced into host cells
18 by the aforementioned techniques. Generally, a plasmid
19 vector is introduced as DNA in a precipitate, such as a
20 calcium phosphate precipitate, or in a complex with a charged
21 lipid. Electroporation also may be used to introduce
22 polynucleotides into a host. If the vector is a virus, it
23 may be packaged in vitro or introduced into a packaging cell
24 and the packaged virus may be transduced into cells. A wide
25 variety of techniques suitable for making polynucleotides and
26 for introducing polynucleotides into cells in accordance with
27 this aspect of the invention are well known and routine to
28 those of skill in the art. Such techniques are reviewed at
29 length in Sambrook et al. cited above, which is illustrative
30 of the many laboratory manuals that detail these techniques.

31 In accordance with this aspect of the invention the
32 vector may be, for example, a plasmid vector, a single or
33 double-stranded phage vector, a single or double-stranded RNA
34 or DNA viral vector. Such vectors may be introduced into
35 cells as polynucleotides, preferably DNA, by well known

1 techniques for introducing DNA and RNA into cells. The
2 vectors, in the case of phage and viral vectors also may be
3 and preferably are introduced into cells as packaged or
4 encapsidated virus by well known techniques for infection and
5 transduction. Viral vectors may be replication competent or
6 replication defective. In the latter case viral propagation
7 generally will occur only in complementing host cells.

8 Preferred among vectors, in certain respects, are those
9 for expression of polynucleotides and polypeptides of the
10 present invention. Generally, such vectors comprise cis-
11 acting control regions effective for expression in a host
12 operatively linked to the polynucleotide to be expressed.
13 Appropriate trans-acting factors either are supplied by the
14 host, supplied by a complementing vector or supplied by the
15 vector itself upon introduction into the host.

16 In certain preferred embodiments in this regard, the
17 vectors provide for specific expression. Such specific
18 expression may be inducible expression or expression only in
19 certain types of cells or both inducible and cell-specific.
20 Particularly preferred among inducible vectors are vectors
21 that can be induced for expression by environmental factors
22 that are easy to manipulate, such as temperature and nutrient
23 additives. A variety of vectors suitable to this aspect of
24 the invention, including constitutive and inducible
25 expression vectors for use in prokaryotic and eukaryotic
26 hosts, are well known and employed routinely by those of
27 skill in the art.

28 The engineered host cells can be cultured in
29 conventional nutrient media, which may be modified as
30 appropriate for, inter alia, activating promoters, selecting
31 transformants or amplifying genes. Culture conditions, such
32 as temperature, pH and the like, previously used with the
33 host cell selected for expression generally will be suitable
34 for expression of polypeptides of the present invention as
35 will be apparent to those of skill in the art.

1 A great variety of expression vectors can be used to
2 express a polypeptide of the invention. Such vectors include
3 chromosomal, episomal and virus-derived vectors e.g., vectors
4 derived from bacterial plasmids, from bacteriophage, from
5 yeast episomes, from yeast chromosomal elements, from viruses
6 such as baculoviruses, papova viruses, such as SV40, vaccinia
7 viruses, adenoviruses, fowl pox viruses, pseudorabies viruses
8 and retroviruses, and vectors derived from combinations
9 thereof, such as those derived from plasmid and bacteriophage
10 genetic elements, such as cosmids and phagemids, all may be
11 used for expression in accordance with this aspect of the
12 present invention. Generally, any vector suitable to
13 maintain, propagate or express polynucleotides to express a
14 polypeptide in a host may be used for expression in this
15 regard.

16 The appropriate DNA sequence may be inserted into the
17 vector by any of a variety of well-known and routine
18 techniques. In general, a DNA sequence for expression is
19 joined to an expression vector by cleaving the DNA sequence
20 and the expression vector with one or more restriction
21 endonucleases and then joining the restriction fragments
22 together using T4 DNA ligase. Procedures for restriction and
23 ligation that can be used to this end are well known and
24 routine to those of skill. Suitable procedures in this
25 regard, and for constructing expression vectors using
26 alternative techniques, which also are well known and routine
27 to those skill, are set forth in great detail in Sambrook et
28 al. cited elsewhere herein.

The DNA sequence in the expression vector is operatively
linked to appropriate expression control sequence(s),
including, for instance, a promoter to direct mRNA
transcription. Representatives of such promoters include the
phage lambda PL promoter, the E. coli lac, trp and tac
promoters, the SV40 early and late promoters and promoters of
retroviral LTRs, to name just a few of the well-known

promoters. It will be understood that numerous promoters not mentioned are suitable for use in this aspect of the invention are well known and readily may be employed by those of skill in the manner illustrated by the discussion and the examples herein.

In general, expression constructs will contain sites for transcription initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, in accordance with many commonly practiced procedures, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast

cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Hosts for of a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention.

More particularly, the present invention also includes recombinant constructs, such as expression constructs, comprising one or more of the sequences described above. The constructs comprise a vector, such as a plasmid or viral vector, into which such a sequence of the invention has been inserted. The sequence may be inserted in a forward or reverse orientation. In certain preferred embodiments in this regard, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and there are many commercially available vectors suitable for use in the present invention.

The following vectors, which are commercially available, are provided by way of example. Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors that are available to those of skill in the art for use in accordance with this aspect of the present invention. It will be appreciated that any other plasmid or vector suitable for, for example, introduction, maintenance, propagation or

1 expression of a polynucleotide or polypeptide of the
2 invention in a host may be used in this aspect of the
3 invention.

4 Promoter regions can be selected from any desired gene
5 using vectors that contain a reporter transcription unit
6 lacking a promoter region, such as a chloramphenicol acetyl
7 transferase ("cat") transcription unit, downstream of
8 restriction site or sites for introducing a candidate
9 promoter fragment; i.e., a fragment that may contain a
10 promoter. As is well known, introduction into the vector of
11 a promoter-containing fragment at the restriction site
12 upstream of the cat gene engenders production of CAT
13 activity, which can be detected by standard CAT assays.
14 Vectors suitable to this end are well known and readily
15 available. Two such vectors are pKK232-2 and pCM7. Thus,
16 promoters for expression of polynucleotides of the present
17 invention include not only well known and readily available
18 promoters, but also promoters that readily may be obtained by
19 the foregoing technique, using a reporter gene.

20 Among known bacterial promoters suitable for expression
21 of polynucleotides and polypeptides in accordance with the
22 present invention are the E. coli lacI and lacZ and
23 promoters, the T3 and T7 promoters, the gpt promoter, the
24 lambda PR, PL promoters and the trp promoter. Among known
25 eukaryotic promoters suitable in this regard are the CMV
26 immediate early promoter, the HSV thymidine kinase promoter,
27 the early and late SV40 promoters, the promoters of
28 retroviral LTRs, such as those of the Rous sarcoma virus
29 ("RSV"), and metallothionein promoters, such as the mouse
30 metallothionein-I promoter.

31 Selection of appropriate vectors and promoters for
32 expression in a host cell is a well known procedure and the
33 requisite techniques for expression vector construction,
34 introduction of the vector into the host and expression in
35 the host are routine skills in the art.

1. The present invention also relates to host cells
2. containing the above-described constructs discussed above.
3. The host cell can be a higher eukaryotic cell, such as a
4. mammalian cell, or a lower eukaryotic cell, such as a yeast
5. cell, or the host cell can be a prokaryotic cell, such as a
6. bacterial cell.

7. Introduction of the construct into the host cell can be
8. effected by calcium phosphate transfection, DEAE-dextran
9. mediated transfection, cationic lipid-mediated transfection,
10. electroporation, transduction, infection or other methods.
11. Such methods are described in many standard laboratory
12. manuals, such as Davis et al. BASIC METHODS IN MOLECULAR
13. BIOLOGY, (1986).

14. Constructs in host cells can be used in a conventional
15. manner to produce the gene product encoded by the recombinant
16. sequence. Alternatively, the polypeptides of the invention
17. can be synthetically produced by conventional peptide
18. synthesizers.

19. Mature proteins can be expressed in mammalian cells,
20. yeast, bacteria, or other cells under the control of
21. appropriate promoters. Cell-free translation systems can
22. also be employed to produce such proteins using RNAs derived
23. from the DNA constructs of the present invention. Appropriate
24. cloning and expression vectors for use with prokaryotic and
25. eukaryotic hosts are described by Sambrook et al., MOLECULAR
26. CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor
27. Laboratory Press, Cold Spring Harbor, N.Y. (1989).

28. Generally, recombinant expression vectors will include
29. origins of replication, a promoter derived from a highly-
30. expressed gene to direct transcription of a downstream
31. structural sequence, and a selectable marker to permit
32. isolation of vector containing cells after exposure to the
33. vector. Among suitable promoters are those derived from the
34. genes that encode glycolytic enzymes such as 3-
35. phosphoglycerate kinase ("PGK"), α -factor, acid phosphatase,

1 and heat shock proteins, among others. Selectable markers
2 include the ampicillin resistance gene of *E. coli* and the
3 *trp1* gene of *S. cerevisiae*.

4 Transcription of the DNA encoding the polypeptides of
5 the present invention by higher eukaryotes may be increased
6 by inserting an enhancer sequence into the vector. Enhancers
7 are cis-acting elements of DNA, usually about from 10 to 300
8 bp that act to increase transcriptional activity of a
9 promoter in a given host cell-type. Examples of enhancers
10 include the SV40 enhancer, which is located on the late side
11 of the replication origin at bp 100 to 270, the
12 cytomegalovirus early promoter enhancer, the polyoma enhancer
13 on the late side of the replication origin, and adenovirus
14 enhancers.

15 Polynucleotides of the invention, encoding the
16 heterologous structural sequence of a polypeptide of the
17 invention generally will be inserted into the vector using
18 standard techniques so that it is operably linked to the
19 promoter for expression. The polynucleotide will be
20 positioned so that the transcription start site is located
21 appropriately 5' to a ribosome binding site. The ribosome
22 binding site will be 5' to the AUG that initiates translation
23 of the polypeptide to be expressed. Generally, there will be
24 no other open reading frames that begin with an initiation
25 codon, usually AUG, and lie between the ribosome binding site
26 and the initiating AUG. Also, generally, there will be a
27 translation stop codon at the end of the polypeptide and
28 there will be a polyadenylation signal and a transcription
29 termination signal appropriately disposed at the 3' end of
30 the transcribed region.

31 For secretion of the translated protein into the lumen
32 of the endoplasmic reticulum, into the periplasmic space or
33 into the extracellular environment, appropriate secretion
34 signals may be incorporated into the expressed polypeptide.

1 The signals may be endogenous to the polypeptide or they may
2 be heterologous signals.

3 The polypeptide may be expressed in a modified form,
4 such as a fusion protein, and may include not only secretion
5 signals but also additional heterologous functional regions.
6 Thus, for instance, a region of additional amino acids,
7 particularly charged amino acids, may be added to the N-
8 terminus of the polypeptide to improve stability and
9 persistence in the host cell, during purification or during
10 subsequent handling and storage. Also, region also may be
11 added to the polypeptide to facilitate purification. Such
12 regions may be removed prior to final preparation of the
13 polypeptide. The addition of peptide moieties to
14 polypeptides to engender secretion or excretion, to improve
15 stability and to facilitate purification, among others, are
16 familiar and routine techniques in the art.

17 Suitable prokaryotic hosts for propagation, maintenance
18 or expression of polynucleotides and polypeptides in
19 accordance with the invention include *Escherichia coli*,
20 *Bacillus subtilis* and *Salmonella typhimurium*. Various
21 species of *Pseudomonas*, *Streptomyces*, and *Staphylococcus* are
22 suitable hosts in this regard. Moreover, many other hosts
23 also known to those of skill may be employed in this regard.

24 As a representative but non-limiting example, useful
25 expression vectors for bacterial use can comprise a
26 selectable marker and bacterial origin of replication derived
27 from commercially available plasmids comprising genetic
28 elements of the well known cloning vector pBR322 (ATCC
29 37017). Such commercial vectors include, for example,
30 pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1
31 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone"
32 sections are combined with an appropriate promoter and the
33 structural sequence to be expressed.

34 Following transformation of a suitable host strain and
35 growth of the host strain to an appropriate cell density,

1 where the selected promoter is inducible it is induced by
2 appropriate means (e.g., temperature shift or exposure to
3 chemical inducer) and cells are cultured for an additional
4 period..

5 Cells typically then are harvested by centrifugation,
6 disrupted by physical or chemical means, and the resulting
7 crude extract retained for further purification.

8 Microbial cells employed in expression of proteins can
9 be disrupted by any convenient method, including freeze-thaw
0 cycling, sonication, mechanical disruption, or use of cell
1 lysing agents, such methods are well know to those skilled in
2 the art.

3 Various mammalian cell culture systems can be employed
4 for expression, as well. Examples of mammalian expression
5 systems include the COS-7 lines of monkey kidney fibroblast,
6 described in Gluzman et al., Cell 23: 175 (1981). Other cell
7 lines capable of expressing a compatible vector include for
8 example, the C127, 3T3, CHO, HeLa, human kidney 293 and BHK
9 cell lines.

0 Mammalian expression vectors will comprise an origin of
1 replication, a suitable promoter and enhancer, and also any
2 necessary ribosome binding sites, polyadenylation sites,
3 splice donor and acceptor sites, transcriptional termination
4 sequences, and 5' flanking non-transcribed sequences that are
5 necessary for expression. In certain preferred embodiments
6 in this regard DNA sequences derived from the SV40 splice
7 sites, and the SV40 polyadenylation sites are used for
8 required non-transcribed genetic elements of these types.

9 The DDCR polypeptide can be recovered and purified from
0 recombinant cell cultures by well-known methods including
1 ammonium sulfate or ethanol precipitation, acid extraction,
2 anion or cation exchange chromatography, phosphocellulose
3 chromatography, hydrophobic interaction chromatography,
4 affinity chromatography, hydroxylapatite chromatography and
5 lectin chromatography. Most preferably, high performance

1 liquid chromatography ("HPLC") is employed for purification.
2 Well known techniques for refolding protein may be employed
3 to regenerate active conformation when the polypeptide is
4 denatured during isolation and or purification.

5 Polypeptides of the present invention include naturally
6 purified products, products of chemical synthetic procedures,
7 and products produced by recombinant techniques from a
8 prokaryotic or eukaryotic host, including, for example,
9 bacterial, yeast, higher plant, insect and mammalian cells.
10 Depending upon the host employed in a recombinant production
11 procedure, the polypeptides of the present invention may be
12 glycosylated or may be non-glycosylated. In addition,
13 polypeptides of the invention may also include an initial
14 modified methionine residue, in some cases as a result of
15 host-mediated processes.

16 DDCR polynucleotides and polypeptides may be used in
17 accordance with the present invention for a variety of
18 applications, particularly those that make use of the
19 chemical and biological properties of DDCR. Among these are
20 applications in treatment of tumors, resistance to parasites,
21 bacteria and viruses, to induce proliferation of T-cells,
22 endothelial cells and certain hematopoietic cells, to treat
23 restenosis, graft vs. host disease, to regulate anti-viral
24 responses and to prevent certain autoimmune diseases after
25 stimulation of DDCR by an agonist. Additional applications
26 relate to diagnosis and to treatment of disorders of cells,
27 tissues and organisms. These aspects of the invention are
28 discussed further below.

29 **DDCR Polypeptides and Fragments**

30 The invention further provides an isolated DDCR
31 polypeptide having the amino acid sequence shown in Figure 1

1 [SEQ ID NO 2], or a fragment thereof. It will be recognized
2 in the art that some amino acid sequence of DDCR can be
3 varied without significant effect of the structure or
4 function of the protein. If such differences in sequence are
5 contemplated, it should be remembered that there will be
6 critical areas on the protein which determine activity. Such
7 areas will usually comprise residues which make up the ligand
8 binding site or the death domain, or which form tertiary
9 structures which affect these domains. In general, it is
10 possible to replace residues which form the tertiary
11 structure, provided that residues performing a similar
12 function are used. In other instances, the type of residue
13 may be completely unimportant if the alteration occurs at a
14 non-critical region of the protein.

15 Thus, the invention further includes variations of the
16 DDCR protein which show substantial DDCR protein activity or
17 which include regions of DDCR such as the protein fragments
18 discussed below. Such mutants include deletions, insertions,
19 inversions, repeats, and type substitutions (for example,
20 substituting one hydrophilic residue for another, but not
21 strongly hydrophilic for strongly hydrophobic as a rule).
22 Small changes or such "neutral" amino acid substitutions will
23 generally have little effect on activity.

24 Typically seen as conservative substitutions are the
25 replacements, one for another, among the aliphatic amino
26 acids Ala, Val, Leu and Ile; interchange of the hydroxyl
27 residues Ser and Thr, exchange of the acidic residues Asp and
28 Glu, substitution between the amide residues Asn and Gln,
29 exchange of the basic residues Lys and Arg and replacements
30 among the aromatic residues Phe, Tyr.

31 The polypeptides of the present invention are preferably
32 provided in an isolated form, and preferably are
33 substantially purified. A recombinantly produced version of
34 the DDCR polypeptide is substantially purified by the one-
35 step method described in Smith and Johnson, *Gene* 67:31-40

1 (1988).

2 The polypeptides of the present invention include the
3 polypeptide of SEQ ID NO:2 (in particular the mature
4 polypeptide) as well as polypeptides which have at least 70%
5 similarity (preferably at least 70% identity) to the
6 polypeptide of SEQ ID NO:2 and more preferably at least 90%
7 similarity (more preferably at least 90% identity) to the
8 polypeptide of SEQ ID NO:2 and still more preferably at least
9 95% similarity (still more preferably at least 95% identity)
10 to the polypeptide of SEQ ID NO:2 and also include portions
11 of such polypeptides with such portion of the polypeptide
12 generally containing at least 30 amino acids and more
13 preferably at least 50 amino acids.

14 As known in the art "similarity" between two
15 polypeptides is determined by comparing the amino acid
16 sequence and its conserved amino acid substitutes of one
17 polypeptide to the sequence of a second polypeptide.

18 The present inventors have discovered that the DDCR
19 polypeptide is a 428 residue protein exhibiting three main
20 structural domains. First, the ligand binding domain was
21 identified within residues from about 30 to about 215 in
22 Figure 1 [SEQ ID NO 2]. Second, the transmembrane domain was
23 identified within residues from about 215 to about 240 in
24 Figure 1 [SEQ ID NO 2]. Third, the intracellular domain was
25 identified within residues from about 240 to about 428 in
26 Figure 1 [SEQ ID NO 2]. Importantly, the intracellular
27 domain includes a death domain at residues from about 350 to
28 about 420. Further preferred fragments of the polypeptide
29 shown in Figure 1 [SEQ ID NO 2] include the mature protein
30 from residues about 30 to about 420.

31 Thus, the invention further provides DDCR polypeptide
32 fragments selected from the mature DDCR protein the DDCR
33 ligand binding domain, the DDCR transmembrane domain, the
34 DDCR intracellular domain, and the DDCR death domain. As
35 described in detail below, such fragments of DDCR can be used

1 to raise polyclonal and monoclonal antibody-agonists and
2 antagonists capable of enhancing or inhibiting the DDCR
3 ligand/receptor signaling pathway. Further, such fragments
4 can be used in the yeast two-hybrid system to "capture" DDCR
5 binding proteins which are also candidate agonist and
6 antagonist according to the present invention. The yeast two
7 hybrid system, which is discussed in more detail below, is
8 described in Fields and Song, *Nature* 340:245-246 (1989).
9 Further, the ligand binding domain in soluble form is itself
10 useful as an antagonist capable of inhibiting DDCR signaling.

11 It will be appreciated by those of skill in the art that
12 other DDCR fragments will also be useful for raising
13 polyclonal and monoclonal antibodies according to the present
14 invention. Such fragments include truncation mutants of the
15 full length or mature DDCR polypeptide. Also preferred in
16 this aspect of the invention are fragments characterized by
17 structural or functional attributes of DDCR. Preferred
18 embodiments of the invention in this regard include fragments
19 that comprise alpha-helix and alpha-helix forming regions
20 ("alpha-regions"), beta-sheet and beta-sheet-forming regions
21 ("beta-regions"), turn and turn-forming regions ("turn-
22 regions"), coil and coil-forming regions ("coil-regions"),
23 hydrophilic regions, hydrophobic regions, alpha amphipathic
24 regions, beta amphipathic regions, flexible regions, surface-
25 forming regions and high antigenic index regions of DDCR.

26 Certain preferred regions in these regards are set out
27 in Figure 3, and include, but are not limited to, regions of
28 the aforementioned types identified by analysis of the amino
29 acid sequence set out in Figure 1. As set out in Figure 3,
30 such preferred regions include Garnier-Robson alpha-regions,
31 beta-regions, turn-regions and coil-regions, Chou-Fasman
32 alpha-regions, beta-regions and turn-regions, Kyte-Doolittle
33 hydrophilic regions and hydrophilic regions, Eisenberg alpha
34 and beta amphipathic regions, Karplus-Schulz flexible
35 regions, Emini surface-forming regions and Jameson-Wolf high

1 antigenic index regions.

2
3 **Polypeptide assays**

4
5 The present invention also relates to diagnostic assays
6 such as quantitative and diagnostic assays for detecting
7 levels of DDCR protein, or the soluble form thereof, in cells
8 and tissues, including determination of normal and abnormal
9 levels. Thus, for instance, a diagnostic assay in accordance
10 with the invention for detecting over-expression of DDCR, or
11 soluble form thereof, compared to normal control tissue
12 samples may be used to detect the presence of tumors, for
13 example. Assay techniques that can be used to determine
14 levels of a protein, such as an DDCR protein of the present
15 invention, or a soluble form thereof, in a sample derived
16 from a host are well-known to those of skill in the art.
17 Such assay methods include radioimmunoassays, competitive-
18 binding assays, Western Blot analysis and ELISA assays. Among
19 these ELISAs frequently are preferred. An ELISA assay
20 initially comprises preparing an antibody specific to DDCR,
21 or soluble form, preferably a monoclonal antibody. In
22 addition a reporter antibody generally is prepared which
23 binds to the monoclonal antibody. The reporter antibody is
24 attached a detectable reagent such as radioactive,
25 fluorescent or enzymatic reagent, in this example horseradish
26 peroxidase enzyme.

27 To carry out an ELISA assay, a sample is removed from a
28 host and incubated on a solid support, e.g. a polystyrene
29 dish, that binds the proteins in the sample. Any free
30 protein binding sites on the dish are then covered by
31 incubating with a non-specific protein such as bovine serum
32 albumin. Next, the monoclonal antibody is incubated in the
33 dish during which time the monoclonal antibodies attach to
34 any DDCR proteins attached to the polystyrene dish. Unbound
35 monoclonal antibody is washed out with buffer. The reporter

antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to DDCR, or soluble receptor. Unattached reporter antibody is then washed out. Reagents for peroxidase activity, including a colorimetric substrate are then added to the dish. Immobilized peroxidase, linked to DDCR through the primary and secondary antibodies, produces a colored reaction product. The amount of color developed in a given time period indicates the amount of DDCR protein, or soluble form, present in the sample. Quantitative results typically are obtained by reference to a standard curve.

A competition assay may be employed wherein antibodies specific to DDCR, or soluble form, attached to a solid support and labeled DDCR and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity of DDCR in the sample.

Therapeutics

The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, antiviral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. et al., (1986) Tumor Necrosis factors: gene structure and biological activities. Cold Spring Harbor Symp. Quant. Biol. 51, 597-609; Beutler, B., and Cerami, A. Annu. Rev. Biochem. 57:505-518 (1988); Old, L.J., Sci. Am. 258: 59-75 (1988); Fiers, W., FEBS Lett. 285:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors, including the DDCR of the present invention. Cells which express the DDCR polypeptide and have a potent cellular response to DDCR ligands include

lymphocytes, fibroblasts, macrophages, synovial cells, and epithelial cells. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (Ameisen, J.C., *AIDS* 8:1197-1213 (1994); Krammer, P.H., et al., *Curr. Opin. Immunol.* 6:279-289 (1994)).

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, such as breast cancer, prostate cancer, and ovarian cancer); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); myelodysplastic syndromes (such as aplastic anemia), ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Thus, in one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DDCR polypeptide an effective amount of an

agonist capable of increasing DDCR mediated signaling. Preferably, DDCR mediated signaling is increased to treat a disease wherein decreased apoptosis is exhibited.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DDCR polypeptide an effective amount of an antagonist capable of decreasing DDCR mediated signaling. Preferably, DDCR mediated signaling is decreased to treat a disease wherein increased apoptosis is exhibited.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in Science, volume 246, pages 181-296 (October 1989). For

1 example, compounds may be contacted with a cell which
2 expresses the receptor polypeptide of the present invention
3 and a second messenger response, e.g. signal transduction or
4 pH changes, may be measured to determine whether the
5 potential compound activates or inhibits the receptor.

6 Another such screening technique involves introducing
7 RNA encoding the receptor into *Xenopus* oocytes to transiently
8 express the receptor. The receptor oocytes may then be
9 contacted with the receptor ligand and a compound to be
10 screened, followed by detection of inhibition or activation
11 of a calcium signal in the case of screening for compounds
12 which are thought to inhibit activation of the receptor.

13 Another screening technique involves expressing in cells
14 a construct wherein the receptor is linked to a phospholipase
15 C or D. Such cells include endothelial cells, smooth muscle
16 cells, embryonic kidney cells, etc. The screening may be
17 accomplished as hereinabove described by detecting activation
18 of the receptor or inhibition of activation of the receptor
19 from the phospholipase signal.

20 Another method involves screening for compounds which
21 inhibit activation of the receptor polypeptide of the present
22 invention antagonists by determining inhibition of binding of
23 labeled ligand to cells which have the receptor on the
24 surface thereof. Such a method involves transfecting a
25 eukaryotic cell with DNA encoding the receptor such that the
26 cell expresses the receptor on its surface and contacting the
27 cell with a compound in the presence of a labeled form of a
28 known ligand. The ligand can be labeled, e.g., by
29 radioactivity. The amount of labeled ligand bound to the
30 receptors is measured, e.g., by measuring radioactivity of
31 the receptors. If the compound binds to the receptor as
32 determined by a reduction of labeled ligand which binds to
33 the receptors, the binding of labeled ligand to the receptor
34 is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307(1992)).

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DDCR polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the DDCR polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor- β , neurotransmitters (such as glutamate, dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonist include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and β -amyloid peptide. (*Science* 267: 1457-

1 1458 (1995)). Further preferred agonist include polyclonal
2 and monoclonal antibodies raised against the DDCR
3 polypeptide, or a fragment thereof. Such agonist antibodies
4 raised against a TNF-family receptor are disclosed in
5 Tartaglia, L.A., et al., *Proc. Natl. Acad. Sci. USA* 88:9292-
6 9296 (1991); and Tartaglia, L.A., and Goeddel, D.V., *J. Biol.*
7 *Chem.* 267(7):4304-4307(1992)). See, also, PCT Application WO
8 94/09137

9 Antagonist according to the present invention include
10 naturally occurring and synthetic compounds such as, for
11 example, the CD40 ligand, neutral amino acids, zinc,
12 estrogen, androgens, viral genes (such as Adenovirus E1B,
13 Baculovirus p35 and IAP, Cowpox virus crmA, Epstein-Barr
14 virus BHRF1, LMP-1, African swine fever virus LMW5-HL, and
15 Herpesvirus v1 34.5), calpain inhibitors, cysteine protease
16 inhibitors, and tumor promoters (such as FMA, Phenobarbital,
17 and α -Hexachlorocyclohexane). Other antagonists include
18 polyclonal and monoclonal antagonist antibodies raised
19 against the DDCR polypeptides or a fragment thereof. Such
20 antagonist antibodies raised against a TNF-family receptor
21 are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol.*
22 *Chem.* 267(7):4304-4307(1992)); and Tartaglia, L.A., et al.,
23 *Cell* 73:213-216 (1993)). See, also, PCT Application WO
24 94/09137

25 Other potential antagonists include antisense molecules.
26 Antisense technology can be used to control gene expression
27 through antisense DNA or RNA or through triple-helix
28 formation. Antisense techniques are discussed, for example,
29 in Okano, J. *Neurochem.* 56: 560 (1991); OLIGODEOXYNUCLEOTIDES
30 AS ANTISENSE INHIBITORS OF GENE EXPRESSION, CRC Press, Boca
31 Raton, FL (1988). Triple helix formation is discussed in,
32 for instance Lee et al., *Nucleic Acids Research* 6: 3073
33 (1979); Cooney et al., *Science* 241: 456 (1988); and Dervan et
34 al., *Science* 251: 1360 (1991). The methods are based on
35 binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into receptor polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed in vivo to inhibit production of the receptor.

Further antagonist according to the present invention include soluble forms of DDCR, i.e., DDCR fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize DDCR mediated signaling by competing with the cell surface DDCR for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting apoptosis induced by TNF-family ligands. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med.*, 182:1395-1401 (1995)).

As indicated polyclonal and monoclonal antibody agonist or antagonist according to the present invention can be raised according to the methods disclosed in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307(1992); Tartaglia, L.A., et al., *Cell* 73:213-216 (1993)), and PCT Application WO 94/09137. The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab'), fragments) which are capable

1 of binding an antigen. Fab and F(ab'), fragments lack the Fc
2 fragment of intact antibody, clear more rapidly from the
3 circulation, and may have less non-specific tissue binding of
4 an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325
5 (1983)).

6 Antibodies according to the present invention may be
7 prepared by any of a variety of methods using DDCR immunogens
8 of the present invention. As indicated, such DDCR immunogens
9 include the full length DDCR polypeptide (which may or may
10 not include the leader sequence) and DDCR polypeptide
11 fragments such as the ligand binding domain, the
12 transmembrane domain, the intracellular domain and the death
13 domain.

14 In a preferred method, antibodies according to the
15 present invention are mAbs. Such mAbs can be prepared using
16 hybridoma technology (Kohler and Millstein, Nature 256:495-
17 497 (1975) and U.S. Patent No. 4,376,110; Harlow et al.,
18 Antibodies: A Laboratory Manual, Cold Spring Harbor
19 Laboratory Press, Cold Spring Harbor, NY, 1988; Monoclonal
20 Antibodies and Hybridomas: A New Dimension in Biological
21 Analyses, Plenum Press, New York, NY, 1980; Campbell,
22 "Monoclonal Antibody Technology," In: Laboratory Techniques
23 in Biochemistry and Molecular Biology, Volume 13 (Burdon et
24 al., eds.), Elsevier, Amsterdam (1984)).

25 In general, such procedures involve immunizing an animal
26 with a cell expressing the DDCR immunogen. The preferred
27 animal for immunization is a mouse. Splenocytes of immunized
28 animals are removed and fused with a suitable myeloma cell
29 line. Any suitable myeloma cell line may be employed in
30 accordance with the present invention. After fusion, the
31 resulting hybridoma cells are selectively maintained in HAT
32 medium, and then cloned by limiting dilution. The hybridoma
33 cells obtained through such a selection are then assayed to
34 identify clones which secrete antibodies capable of binding
35 the DDCR immunogen.

1 For replication, the hybridoma cells of this invention
2 may be cultivated *in vitro* or *in vivo*. Production of high
3 titers of mAbs *in vivo* production makes this the presently
4 preferred method of production. Briefly, cells from the
5 individual hybridomas are injected intraperitoneally into
6 pristane-primed BALB/c mice to produce ascites fluid
7 containing high concentrations of the desired mAbs. Mabs of
8 isotype IgM or IgG may be purified from such ascites fluids,
9 or from culture supernatants, using column chromatography
10 methods well known to those of skill in the art.

Also intended within the scope of the present invention
are humanized chimeric antibodies, produced using genetic
constructs derived from hybridoma cells producing the mAbs
described above. Methods for production of chimeric
antibodies are known in the art. See, for review: Morrison,
Science, 229:1202-1207 (1985); Oi et al., *BioTechniques* 4:214
(1986); see, also: Cabilly et al., U.S. Patent 4,816,567
(3/28/89); Taniguchi et al., EPO Patent Public. EP171496
(2/19/86); Morrison et al., EPO Patent Pub. EP173494
(3/5/86); Neuberger et al., PCT Pub. WO8601533 (3/13/86);
Robinson et al., PCT Pub. WO 8702671 (5/7/87); Boulianne
et al., *Nature* 312:643-646 (1984); Neuberger et al., *Nature*
314:268-270 (1985).

Proteins and other compounds which bind the DDCR domains
are also candidate agonist and antagonist according to the
present invention. Such binding compounds can be "captured"
using the yeast two hybrid system (Fields and Song, *Nature*
340:245-246 (1989)). A modified version of the yeast two-
hybrid system has been described by Roger Brent and his
colleagues (Gyuris, J. et al., *Cell* 75:791-803 (1993);
Zervos, A.S. et al., *Cell* 72:223-232 (1993)). Briefly, a
domain of the DDCR polypeptide is used as bait for binding
compounds. Positives are then selected by their ability to
grow on plates lacking leucine, and then further tested for
their ability to turn blue on plates with X-gal, as

1 previously described in great detail (Gyuris, J. et al., *Cell*
2 75:791-803 (1993)). Preferably, the yeast two-hybrid system
3 is used according to the present invention to capture
4 compounds which bind to either the DDCR ligand binding domain
5 or to the DDCR intracellular domain. Such compounds are good
6 candidate agonist and antagonist of the present invention.
7 This system has been used previously to isolate proteins
8 which bind to the intracellular domain of the p55 and p75 TNF
9 receptors (WO 95/31544).

0 By a "TNF-family ligand" is intended naturally occurring,
1 recombinant, and synthetic ligands that are capable of
2 binding to a member of the TNF receptor family and inducing
3 the ligand/receptor signaling pathway. Members of the TNF
4 ligand family include, but are not limited to, the DDCR
5 ligand, TNF- α , lymphotoxin- α (LT- α , also known as TNF- β), LT-
6 β (found in complex heterotrimer LT- α 2- β), FasL, CD40, CD27,
7 CD30, 4-1BB, OX40 and nerve growth factor (NGF).

8 Representative therapeutic applications of the present
9 invention are discussed in more detail below. The state of
0 immunodeficiency that defines AIDS is secondary to a decrease
1 in the number and function of CD4⁺ T-lymphocytes. Recent
2 reports estimate the daily loss of CD4⁺ T cells to be between
3 3.5×10^7 and 2×10^9 cells (Wei X., et al., *Nature* 373: 117-
4 122(1995)). One cause of CD4⁺ T cell depletion in the setting
5 of HIV infection is believed to be HIV-induced apoptosis.
6 Indeed, HIV-induced apoptotic cell death has been
7 demonstrated not only in vitro but also, more importantly, in
8 infected individuals (Ameisen, J.C., *AIDS* 8:1197-1213 (1994);
9 Finkel, T.H., and Banda, N.K., *Curr. Opin. Immunol.* 6:605-
0 615(1995); Muro-Cacho, C.A. et al., *J. Immunol.* 154:5555-5566
1 (1995)). Furthermore, apoptosis and CD4⁺ T-lymphocyte
2 depletion is tightly correlated in different animal models of
3 AIDS (Brunner, T., et al., *Nature* 373: 441-444 (1995);
4 Gougeon, M.L., et al., *AIDS Res. Hum. Retroviruses* 9:553-563
5 (1993)) and, apoptosis is not observed in those animal models

1 in which viral replication does not result in AIDS (Gougeon,
2 M.L., et al., *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)).
3 Further data indicates that uninfected but primed or
4 activated T lymphocytes from HIV-infected individuals undergo
5 apoptosis after encountering the TNF-family ligand FasL.
6 Using monocytic cell lines that result in death following HIV
7 infection, it has been demonstrated that infection of U937
8 cells with HIV results in the de novo expression of FasL and
9 that FasL mediates HIV-induced apoptosis (Badley, A.D., et
10 al., *J. Virol.* 70:199-206 (1996)). Further the TNF-family
11 ligand was detectable in uninfected macrophages and its
12 expression was upregulated following HIV infection resulting
13 in selective killing of uninfected CD4 T-lymphocytes (Badley,
14 A.D., et al., *J. Virol.* 70:199-206 (1996)). Thus, by the
15 invention, a method for treating HIV individuals is provided
16 which involves administering an antagonist of the present
17 invention to reduce selective killing of CD4 T-lymphocytes.
18 Modes of administration and dosages are discussed in detail
19 below.

20 In rejection of an allograft, the immune system of the
21 recipient animal has not previously been primed to respond
22 because the immune system for the most part is only primed by
23 environmental antigens. Tissues from other members of the
24 same species have not been presented in the same way that,
25 for example, viruses and bacteria have been presented. In
26 the case of allograft rejection, immunosuppressive regimens
27 are designed to prevent the immune system from reaching the
28 effector stage. However, the immune profile of xenograft
29 rejection may resemble disease recurrence more than allograft
30 rejection. In the case of disease recurrence, the immune
31 system has already been activated, as evidenced by
32 destruction of the native islet cells. Therefore, in disease
33 recurrence the immune system is already at the effector
34 stage. Antagonist of the present invention are able to suppress
35 the immune response to both allografts and xenografts because

lymphocytes activated and differentiated into effector cells will express the DDCR polypeptide, and thereby are susceptible to compounds which enhance apoptosis. Thus, the present invention further provides a method for creating immune privileged tissues. Antagonist of the invention can further be used in the treatment of Inflammatory Bowel Disease.

The agonist or antagonists described herein can be administered in vitro, ex vivo, or in vivo to cells which express the receptor of the present invention. By administration of an "effective amount" of an agonist or antagonist is intended an amount of the compound that is sufficient to enhance or inhibit a cellular response to a TNF-family ligand. In particular, by administration of an "effective amount" of an agonist or antagonists is intended an amount effective to enhance or inhibit DDCR mediated apoptosis. Of course, where apoptosis is to be enhanced, an agonist according to the present invention can be co-administered with a TNF-family ligand. One of ordinary skill will appreciate that effective amounts of an agonist or antagonist can be determined empirically and may be employed in pure form or, where such forms exist, in pharmaceutically acceptable salt, ester or prodrug form. The agonist or antagonist may be administered in compositions in combination with one or more pharmaceutically acceptable excipients. It will be understood that, when administered to a human patient, the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgement. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the type and degree of the cellular response to be achieved; activity of the specific agonist or antagonist employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the agonist or antagonist; the

duration of the treatment; drugs used in combination or coincidental with the specific agonist or antagonist; and like factors well known in the medical arts.

For example, satisfactory results are obtained by oral administration of a antagonist or agonist at dosages on the order of from 0.05 to 10 mg/kg/day, preferably 0.1 to 7.5 mg/kg/day, more preferably 0.1 to 2 mg/kg/day, administered once or, in divided doses, 2 to 4 times per day. On administration parenterally, for example by i.v. drip or infusion, dosages on the order of from 0.01 to 5 mg/kg/day, preferably 0.05 to 1.0 mg/kg/day and more preferably 0.1 to 1.0 mg/kg/day can be used. Suitable daily dosages for patients are thus on the order of from 2.5 to 500 mg p.o., preferably 5 to 250 mg p.o., more preferably 5 to 100 mg p.o., or on the order of from 0.5 to 250 mg i.v., preferably 2.5 to 125 mg i.v. and more preferably 2.5 to 50 mg i.v.

Dosaging may also be arranged in a patient specific manner to provide a predetermined concentration of an agonist or antagonist in the blood, as determined by the RIA technique. Thus patient dosaging may be adjusted to achieve regular on-going trough blood levels, as measured by RIA, on the order of from 50 to 1000 ng/ml; preferably 150 to 500 ng/ml.

From above, pharmaceutical compositions are provided comprising an agonist or antagonist and a pharmaceutically acceptable carrier or excipient, which may be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. Importantly, by co-administering an agonist and a TNF-family ligand, clinical side effects can be reduced by using lower doses of both the ligand and the agonist. It will be understood that the agonist can be "co-administered" either before, after, or simultaneously with the TNF-family ligand, depending on the exigencies of a

particular therapeutic application. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

The compositions of the present invention may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of the agonist or antagonist, it is desirable to slow the absorption

from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compounds are mixed with at least one item pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid,

certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame

oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth, and mixtures thereof.

Topical administration includes administration to the skin or mucosa, including surfaces of the lung and eye. Compositions for topical administration, including those for inhalation, may be prepared as a dry powder which may be pressurized or non-pressurized. In nonpressurized powder compositions, the active ingredient in finely divided form may be used in admixture with a larger-sized pharmaceutically acceptable inert carrier comprising particles having a size, for example, of up to 100 μm in diameter. Suitable inert carriers include sugars such as lactose. Desirably, at least 95% by weight of the particles of the active ingredient have an effective particle size in the range of 0.01 to 10 μm .

Alternatively, the composition may be pressurized and contain a compressed gas, such as nitrogen or a liquefied gas propellant. The liquefied propellant medium and indeed the total composition is preferably such that the active ingredient does not dissolve therein to any substantial extent. The pressurized composition may also contain a surface active agent. The surface active agent may be a liquid or solid non-ionic surface active agent or may be a solid anionic surface active agent. It is preferred to use the solid anionic surface active agent in the form of a sodium salt.

Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the agonist or antagonist with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at room temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the drugs.

The agonist or antagonist can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to the agonist or antagonist, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholesterols (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. See, for example, Prescott, Ed., *Methods in Cell Biology*, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 et seq.

Gene therapy

The DDCR polynucleotides, soluble form of the receptor polypeptides, agonists and antagonists that are polypeptides may be employed in accordance with the present invention by expression of such polypeptides in vivo, in treatment modalities often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide, such as a DNA or RNA, encoding a polypeptide ex vivo, and the engineered cells then can be provided to a patient to be treated with the polypeptide. For example, cells may be engineered ex vivo by

the use of a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention. Such methods are well-known in the art and their use in the present invention will be apparent from the teachings herein.

Similarly, cells may be engineered in vivo for expression of a polypeptide in vivo by procedures known in the art. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct then may be isolated and introduced into a packaging cell is transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a patient for engineering cells in vivo and expression of the polypeptide in vivo. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention.

Retroviruses from which the retroviral plasmid vectors herein above mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

Such vectors well include one or more promoters for expressing the polypeptide. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller et al., Biotechniques 7: 980-990 (1989), or any other promoter (e.g., cellular promoters such

as eukaryotic cellular promoters including, but not limited to, the histone, RNA polymerase III, and β -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention will be placed under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoA1 promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs herein above described); the β -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, Y-2, Y-AM, PA12, T19-14X, VT-19-17-H2, YCRE, YCRIP, GP+E-86, GP+envAml2, and DAN cell lines as described in Miller, A. Human Gene Therapy 1: 5-14 (1990). The vector may be transduced into the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO4 precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a

host.

The producer cell line will generate infectious retroviral vector particles, which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

EXAMPLES

The present invention is further described by the following examples. The examples are provided solely to illustrate the invention by reference to specific embodiments. These exemplification's, while illustrating certain specific aspects of the invention, do not portray the limitations or circumscribe the scope of the disclosed invention.

All examples were carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. Routine molecular biology techniques of the following examples can be carried out as described in standard laboratory manuals, such as Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), herein referred to as "Sambrook."

All parts or amounts set out in the following examples are by weight, unless otherwise specified.

Unless otherwise stated size separation of fragments in the examples below was carried out using standard techniques of agarose and polyacrylamide gel electrophoresis ("PAGE") in

Sambrook and numerous other references such as, for instance, by Goeddel et al., Nucleic Acids Res. 8:4057 (1980).

Unless described otherwise, ligations were accomplished using standard buffers, incubation temperatures and times, approximately equimolar amounts of the DNA fragments to be ligated and approximately 10 units of T4 DNA ligase ("ligase") per 0.5 ug of DNA.

Example 1: Expression in *E. coli*

The following primers are used for expression of DDCR extracellular domain in *E. coli*. 5' primer GCGCCATGGGGCCGGCGGCAG contains an NcoI site and 15 nucleotide starting from 290 nucleotide to 304 Figure 1. 3' primer GCGAAGCTTCTAGGACCCAGAACATCTGCC contains a HindIII site, a stop codon and 18 nucleotides complimentary to nucleotide from 822 to 840 in Figure 1. Vector is pQE60. The protein is not tagged.

Example 2: Expression in Mammalian Cells (CHO, COS and Others).

Most of the vectors used for the transient expression of a given gene sequence in mammalian cells carry the SV40 origin of replication. This allows the replication of the vector to high copy numbers in cells (e.g. COS cells) which express the T antigen required for the initiation of viral DNA synthesis. Any other mammalian cell line can also be utilized for this purpose.

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and

late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g. RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, also cellular signals can be used (e.g. human actin, promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC67109). Mammalian host cells that could be used include, human Hela, 283, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1 African green monkey cells, quail QC1-3 cells, mouse L cells and Chinese hamster ovary cells such as

Alternatively, a gene of interest can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) is a useful marker to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Using this marker, the mammalian cells are grown in increasing amounts of methotrexate for selection and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) cells are often used for the production of proteins.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., Molecular and Cellular Biology, March 1985, 438-4470) plus a fragment of the CMV-enhancer (Boshart et al., Cell 41, 521-530 [1985]). Multiple cloning sites, e.g. with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors

contain in addition the 5' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Example 2A: Expression of extracellular soluble domain of DDCR in COS cells

The expression plasmid, DDCR HA, is made by cloning a cDNA encoding DDCR into the expression vector pcDNA1/Amp (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNA1/Amp contains: (1) an E.coli origin of replication effective for propagation in E. coli and other prokaryotic cell; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron, and a polyadenylation signal arranged so that a cDNA conveniently can be placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker.

A DNA fragment encoding the entire DDCR precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson et al., Cell 37: 767 (1984). The fusion of the HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is as follows:

The DDCR cDNA of the deposit clone is amplified using primers that contained convenient restriction sites, much as described above regarding the construction of expression vectors for expression of DDCR in E. coli and S. fugiperda.

To facilitate detection, purification and character-

ization of the expressed DDCR, one of the primers contains a hemagglutinin tag ('HA tag') as described above.

Suitable primers include that following, which is used in this example: the 5' primer, 5' CGCGGATCCATGGAGGAGACCGCAGCAG 3' contains the underlined BamHI site, an ATG start codon and 5 codons thereafter.

The 3' primer, containing the underlined XbaI site, stop codon, hemagglutinin tag and last 14 nucleotide of 3' coding sequence (at the 3' end) has the following sequence: 5' GCGTCTAGATCAAAGCGTAGTCTGGGACGTCGTATGGGTACGGGCCGCGCTGCA 3'.

The PCR amplified DNA fragment and the vector, pcDNA1/Amp, are digested with BamHI and XbaI and then ligated. The ligation mixture is transformed into E. coli strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis and gel sizing for the presence of the DDCR-encoding fragment.

For expression of recombinant DDCP, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989).

Cells are incubated under conditions for expression of DDCR by the vector.

Expression of the DDCR HA fusion protein is detected by radiolabelling and immunoprecipitation, using methods described in, for example Harlow et al., ANTIBODIES: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing 35S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and the

lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson et al. cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE gels and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 2B: Expression and purification of human DDCR using the CHO Expression System

The DNA sequence encoding DDCR in the deposited polynucleotide is amplified using PCR oligonucleotide primers specific to the amino acid carboxyl terminal sequence of the DDCR protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The 5' oligonucleotide primer has the sequence 5'-CGCGGATCCATGGAGGAGACGCGAGCAG-3' containing the underlined BamHI restriction site, which encodes a start AUG, followed by 15 nucleotides of the DDCR coding sequence set out in Figure 1 beginning with the 1st base of the ATG codon.

The 3' primer has the sequence 5'-CGCGGATCTCAGGGCCGCTGCA-3' containing the underlined BamHI restriction site followed by 17 nucleotides complementary to the last 14 nucleotides of the DDCR coding sequence set out in Figure 1, plus the stop codon.

The restriction sites are convenient to restriction enzyme sites in the CHO expression vectors PC4.

The amplified DDCR DNA and the vector PC4 both are digested with BamHI and the digested DNAs then ligated together. Insertion of the DDCR DNA into the BamHI

restricted vector placed the DDCR coding region downstream of and operably linked to the vector's promoter.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y. (1989).

Example 3: Cloning and expression of the soluble extracellular domain of DDCR in a baculovirus expression system

The cDNA sequence encoding the soluble extracellular domain of DDCR protein in the deposited clone is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' CGCGGATCCCAGCCGAGG TGTGACTGTGCCGTGACTTCCACAAGAAG 3' containing the underlined Bam HI restriction enzyme site followed by Kozak sequence and a number of bases of the sequence of DDCR of Figure 1. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DDCR provides an efficient signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.* 196: 947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer has the sequence 5' GCGAGATCTAGTCTGACCC AGAATCTGCTCC 3' containing the underlined XbaI restriction followed by nucleotides complementary to the DDCR nucleotide sequence set out in Figure 1, followed by the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with

1 BamHI and Asp718 and again is purified on a 1% agarose gel.
2 This fragment is designated herein F2.

3 The vector pA2GP is used to express the DDCR protein in
4 the baculovirus expression system, using standard methods,
5 such as those described in Summers et al, A MANUAL OF METHODS
6 FOR BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES,
7 Texas Agricultural Experimental Station Bulletin No. 1555
8 (1987). This expression vector contains the strong
9 polyhedrin promoter of the Autograph californica nuclear
10 polyhedrosis virus (AcMNPV) followed by convenient
11 restriction sites. For an easy selection of recombinant
12 virus the beta-galactosidase gene from E.coli is inserted in
13 the same orientation as the polyhedrin promoter and is
14 followed by the polyadenylation signal of the polyhedrin
15 gene. The polyhedrin sequences are flanked at both sides by
16 viral sequences for cell-mediated homologous recombination
17 with wild-type viral DNA to generate viable virus that
18 express the cloned polynucleotide.

19 Many other baculovirus vectors could be used in place of
20 pA2GP, such as pAc373, pVL941 and pAcIM1 provided, as those
21 of skill readily will appreciate, that construction provides
22 appropriately located signals for transcription, translation,
23 trafficking and the like, such as an in-frame AUG and a
24 signal peptide, as required. Such vectors are described in
25 Luckow et al., Virology 170: 31-39, among others.

26 The plasmid is digested with the restriction enzymes Bam
27 HI and XbaI and then is dephosphorylated using calf
28 intestinal phosphatase, using routine procedures known in the
29 art. The DNA is then isolated from a 1% agarose gel using a
30 commercially available kit ("GeneClean" BIO 101 Inc., La
31 Jolla, Ca.). This vector DNA is designated herein "V2".

32 Fragment F2 and the dephosphorylated plasmid V2 are
33 ligated together with T4 DNA ligase. E.coli HB101 cells are
34 transformed with ligation mix and spread on culture plates.
35 Bacteria are identified that contain the plasmid with the

1 human DDCR gene by digesting DNA from individual colonies
2 using Bam HI and XbaI and then analyzing the digestion
3 product by gel electrophoresis. The sequence of the cloned
4 fragment is confirmed by DNA sequencing. This plasmid is
5 designated herein pBacDDCR.

6 5 µg of the plasmid pBacDDCR is co-transfected with 1.0
7 µg of a commercially available linearized baculovirus DNA
8 ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.),
9 using the lipofection method described by Felgner et al.,
10 Proc. Natl. Acad. Sci. USA 84: 7413-7417 (1987). 1 µg of
11 BaculoGold™ virus DNA and 5 µg of the plasmid pBacDDCR are
12 mixed in a sterile well of a microtiter plate containing 50
13 µl of serum free Grace's medium (Life Technologies Inc.,
14 Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl
15 Grace's medium are added, mixed and incubated for 15 minutes
16 at room temperature. Then the transfection mixture is added
17 drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35
18 mm tissue culture plate with 1 ml Grace's medium without
19 serum. The plate is rocked back and forth to mix the newly
20 added solution. The plate is then incubated for 7 hours at
21 27°C. After 5 hours the transfection solution is removed from
22 the plate and 1 ml of Grace's insect medium supplemented with
23 10% fetal calf serum is added. The plate is put back into an
24 incubator and cultivation is continued at 27°C for four days.

25 After four days the supernatant is collected and a
26 plaque assay is performed, as described by Summers and Smith,
27 cited above. An agarose gel with "Blue Gal" (Life
28 Technologies Inc., Gaithersburg) is used to allow easy
29 identification and isolation of gal-expressing clones, which
30 produce blue-stained plaques. (A detailed description of a
31 "plaque assay" of this type can also be found in the user's
32 guide for insect cell culture and baculovirology distributed
33 by Life Technologies Inc., Gaithersburg, page 9-10).

34 Four days after serial dilution, the virus is added to
35 the cells. After appropriate incubation, blue stained

1 plaques are picked with the tip of an Eppendorf pipette. The
2 agar containing the recombinant viruses is then resuspended
3 in an Eppendorf tube containing 200 μ l of Grace's medium.
4 The agar is removed by a brief centrifugation and the
5 supernatant containing the recombinant baculovirus is used to
6 infect Sf9 cells seeded in 35 mm dishes. Four days later the
7 supernatants of these culture dishes are harvested and then
8 they are stored at 4°C. A clone containing properly inserted
9 DDCR is identified by DNA analysis including restriction
10 mapping and sequencing. This is designated herein as V-DDCR.

11 Sf9 cells are grown in Grace's medium supplemented with
12 10% heat-inactivated FBS. The cells are infected with the
13 recombinant baculovirus V-DDCR at a multiplicity of infection
14 ("MOI") of about 2 (about 1 to about 3). Six hours later the
15 medium is removed and is replaced with SF900 II medium minus
16 methionine and cysteine (available from Life Technologies
17 Inc., Gaithersburg). 42 hours later, 5 μ Ci of 35S-methionine
18 and 5 μ Ci 35S cysteine (available from Amersham) are added.
19 The cells are further incubated for 16 hours and then they
20 are harvested by centrifugation, lysed and the labeled
21 proteins are visualized by SDS-PAGE and autoradiography.

25 **Example 4: Tissue distribution of DDCR expression**

27 Northern blot analysis is carried out to examine the
28 levels of expression of DDCR in human tissues, using methods
29 described by, among others, Sambrook et al. cited above.
30 Total cellular RNA samples are isolated with RNazol™ B system
31 (Biotech Laboratories, Inc. 6023 South Loop East, Houston, TX
32 77033).

33 About 10 μ g of Total RNA is isolated from tissue samples.
34 The RNA is size resolved by electrophoresis through a 1%
35 agarose gel under strongly denaturing conditions. RNA is

1 blotted from the gel onto a nylon filter, and the filter then
2 is prepared for hybridization to a detectably labeled
3 polynucleotide probe.

4 As a probe to detect mRNA that encodes DDCR, the
5 antisense strand of the coding region of the cDNA insert in
6 the deposited clone is labeled to a high specific activity.
7 The cDNA is labeled by primer extension, using the Prime-It
8 kit, available from Stratagene. The reaction is carried out
9 using 50 ng of the cDNA, following the standard reaction
10 protocol as recommended by the supplier. The labeled
11 polynucleotide is purified away from other labeled reaction
12 components by column chromatography using a Select-G-50
13 column, obtained from 5-Prime - 3-Prime, Inc. of 5603
14 Arapahoe Road, Boulder, CO 80303.

15 The labeled probe is hybridized to the filter, at a
16 concentration of 1,000,000 cpm/ml, in a small volume of 7%
17 SDS, 0.5 M NaPO₄, pH 7.4 at 65°C, overnight.

18 Thereafter the probe solution is drained and the filter
19 is washed twice at room temperature and twice at 60°C with 0.5
20 x SSC, 0.1% SDS. The filter then is dried and exposed to
21 film at -70°C overnight with an intensifying screen.

22
23 **Example 5: Gene therapeutic expression of human DDCR**

24
25 Fibroblasts are obtained from a subject by skin biopsy.
26 The resulting tissue is placed in tissue-culture medium and
27 separated into small pieces. Small chunks of the tissue are
28 placed on a wet surface of a tissue culture flask,
29 approximately ten pieces are placed in each flask. The flask
30 is turned upside down, closed tight and left at room
31 temperature overnight. After 24 hours at room temperature,
32 the flask is inverted - the chunks of tissue remain fixed to
33 the bottom of the flask - and fresh media is added (e.g.,
34 Ham's F12 media, with 10% FBS, penicillin and streptomycin).
35 The tissue is then incubated at 37°C for approximately one

1 week. At this time, fresh media is added and subsequently
2 changed every several days. After an additional two weeks in
3 culture, a monolayer of fibroblasts emerges. The monolayer
4 is trypsinized and scaled into larger flasks.

5 A vector for gene therapy is digested with restriction
6 enzymes for cloning a fragment to be expressed. The digested
7 vector is treated with calf intestinal phosphatase to prevent
8 self-ligation. The dephosphorylated, linear vector is
9 fractionated on an agarose gel and purified.

10 DDCR cDNA capable of expressing active DDCR, is
11 isolated. The ends of the fragment are modified, if
12 necessary, for cloning into the vector. For instance, 5'
13 overhanging may be treated with DNA polymerase to create
14 blunt ends. 3' overhanging ends may be removed using S1
15 nuclease. Linkers may be ligated to blunt ends with T4 DNA
16 ligase.

17 Equal quantities of the Moloney murine leukemia virus
18 linear backbone and the DDCR fragment are mixed together and
19 joined using T4 DNA ligase. The ligation mixture is used to
20 transform E. Coli and the bacteria are then plated onto agar-
21 containing kanamycin. Kanamycin phenotype and restriction
22 analysis confirm that the vector has the properly inserted
23 gene.

24 Packaging cells are grown in tissue culture to confluent
25 density in Dulbecco's Modified Eagles Medium (DMEM) with 10%
26 calf serum (CS), penicillin and streptomycin. The vector
27 containing the DDCR gene is introduced into the packaging
28 cells by standard techniques. Infectious viral particles
29 containing the DDCR gene are collected from the packaging
30 cells, which now are called producer cells.

31 Fresh media is added to the producer cells, and after an
32 appropriate incubation period media is harvested from the
33 plates of confluent producer cells. The media, containing
34 the infectious viral particles, is filtered through a
35 Millipore filter to remove detached producer cells. The

1 filtered media then is used to infect fibroblast cells.
2 Media is removed from a sub-confluent plate of fibroblasts
3 and quickly replaced with the filtered media. Polybrene
4 (Aldrich) may be included in the media to facilitate
5 transduction. After appropriate incubation, the media is
6 removed and replaced with fresh media. If the titer of virus
7 is high, then virtually all fibroblasts will be infected and
8 no selection is required. If the titer is low, then it is
9 necessary to use a retroviral vector that has a selectable
10 marker, such as neo or his, to select out transduced cells
11 for expansion.

12 Engineered fibroblasts then may be injected into rats.
13 either alone or after having been grown to confluence on
14 microcarrier beads, such as cytodex 3 beads. The injected
15 fibroblasts produce DDCR product, and the biological actions
16 of the protein are conveyed to the host.

17 It will be clear that the invention may be practiced
18 otherwise than as particularly described in the foregoing
19 description and examples.

20 Numerous modifications and variations of the present
21 invention are possible in light of the above teachings and,
22 therefore, are within the scope of the appended claims.
23

24 The disclosures of all patents, patent applications, and
25 publications referred to herein are hereby incorporated by
26 reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: YU, GUO-LIANG
NI, JIAN
GENTZ, REINER L
- (ii) TITLE OF INVENTION: DEATH DOMAIN CONTAINING RECEPTOR
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN, CECCHI,
STEWART & OLSTEIN
(B) STREET: 6 BECKER FARM ROAD
(C) CITY: ROSELAND
(D) STATE: NEW JERSEY
(E) COUNTRY: USA
(F) ZIP: 07068-1739
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ferraro, Gregory D
(B) REGISTRATION NUMBER: 36,134
(C) REFERENCE/DOCKET NUMBER: 325800-553
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-994-1700
(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 198..1481

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATGGGTGGG GGTGGGGGCG CTGCTGGATT CCGCTCTCG TGGATGGGAA ACTTGTGAGG	60
GGCTGGTAAG CCCCCCTCC GAAGCCTGGT GTGTGCGCGG GCGGAAGGAA GTTAGTTTCC	120
TTCTCACCCA TGGGCACCCC TTCTGCCCGG GGCTGGGAA GTGGGCTGCT CTGTGGGCAA	180

-ATGCTGGGGC CTCTGAA	ATG GAG GAG ACG CAG CAG GGA GAG GCC CCA CGT	230
	Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg	
	1 5 10	
GGG CAG CTG CCG GGA GAG TCA GCA GCA CCT GTC CCC CAG GCG CTC CTC	278	
Gly Gln Leu Arg Gly Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu		
	15 20 25	
CTG GTG CTG CTG GGG GCC CCG GCC CAG GGC GGC ACT CGT AGC CCC AGG	326	
Leu Val Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg		
	30 35 40	
TGT GAC TGT GCC GGT GAC TTC CAC AAG AAG ATT GGT CTG TTT TGT TGC	374	
Cys Asp Cys Ala Gly Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys		
	45 50 55	
AGA GGC TGC CCA GCG GGG CAC TAC CTG AAG GCC CCT TGC ACG GAG CCC	422	
Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro		
	60 65 70 75	
TGC GGC AAC TCC ACC TGC CTT GTG TGT CCC CAA GAC ACC TTC TTG GCC	470	
Cys Gly Asn Ser Thr Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala		
	80 85 90	
TGG GAG AAC CAC CAT AAT TCT GAA TGT GCC CCG TGC CAG GCC TGT GAT	518	
Trp Glu Asn His His Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp		
	95 100 105	
GAG CAG GCC TCC CAG GTG GCG CTG GAG AAC TGT TCA GCA GTG GCC GAC	566	
Glu Gln Ala Ser Gln Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp		
	110 115 120	
ACC CCG TGT GGC TGT AAG CCA GGT TGG TTT GTG GAG TGC CAG GTC AGC	614	
Thr Arg Cys Gly Cys Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser		
	125 130 135	
CAA TGT GTC AGC AGT TCA CCC TTC TAC TGC CAA CCA TGC CTA GAC TGC	662	
Gln Cys Val Ser Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys		
	140 145 150 155	
GGG GCC CTG CAC CCG CAC ACA CCG CTA CTC TGT TCC CCG AGA GAT ACT	710	
Gly Ala Leu His Arg His Thr Arg Leu Cys Ser Arg Arg Asp Thr		
	160 165 170	
GAC TGT GGG ACC TGC CTG CCT GGC TTC TAT GAA CAT GGC GAT GGC TGC	758	
Asp Cys Gly Thr Cys Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys		
	175 180 185	
GTG TCC TGC CCC ACG AGC ACC CTG GGG AGC TGT CCA GAG CCG TGT GCC	806	
Val Ser Cys Pro Thr Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala		
	190 195 200	
GCT GTC TGT GGC TGG AGG CAG ATG TTC TGG GTC CAG GTG CTC CTG GCT	854	
Ala Val Cys Gly Trp Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala		
	205 210 215	
GGC CTT GTG GTC CCC CTC CTG CTT GGG GGC ACC CTT GAC CTA CAC ATA	902	
Gly Leu Val Val Pro Leu Leu Leu Gly Gly Thr Leu Asp Leu His Ile		
	220 225 230 235	
CCG CCA CTG CTG GCT CAC AAG CCC CTG GTT ACT CCA GAT GAA GCT GGG	950	
Pro Pro Leu Leu Ala His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly		
	240 245 250	
ATG GAG GCT CTG AAC CCA CCA CCG GGC ACC CAT CTG TCA CCC TTG GAC	998	
Met Glu Ala Leu Asn Pro Pro Pro Gly Thr His Leu Ser Pro Leu Asp		
	255 260 265	

AGC CCC CAC ACC CTT CTA GCA CCT CCT GAC AGC AGT GAG AAG ATC TGC Ser Ala His Thr Leu Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys 270 275 280	1046
ACC GTC CAG TTG GTG GGT AAC AGC TGG ACC CCT GGC TAC CCC GAG ACC Thr Val Gln Leu Val Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr 285 290 295	1094
CAG GAG GCG CTC TGC CCG CAG GTG ACA TGG TCC TGG GAC CAG TTG CCC Gln Glu Ala Leu Cys Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro 300 305 310 315	1142
AGC AGA GCT CTT GGC CCC GCT GCT GCG CCC ACA CTC TCG CCA GAG TCC Ser Arg Ala Leu Gly Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser 320 325 330	1190
CCA GCC GGC TCG CCA GCC ATG ATG CTG CAG CCG GGC CCG CAG CTC TAC Pro Ala Gly Ser Pro Ala Met Met Leu Gln Pro Gly Pro Glu Leu Tyr 335 340 345	1238
GAC GTG ATG GAC GCG GTC CCA GCG CGG CGC TGG AAG GAG TTC GTG CGC Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg 350 355 360	1286
ACG CTG GGG CTG CGC GAG GCA GAG ATC GAA GCC GTG GAG GTG GAG ATC Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile 365 370 375	1334
GGC CGC TTC CGA GAC TAG CAG TAC GAG ATG CTC AAG CGC TGG CGC CAG Gly Arg Phe Ser Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln 380 385 390 395	1382
CAG CAG CCC GCG GGC CTC GGA GCC GTT TAC GCG GCC CTG GAG CGC ATG Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met 400 405 410	1430
GGG CTG GAC GGC TGC GTG GAA GAC TTG CGC AGC CGC CTG CAG CGC GGC Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly 415 420 425	1478
CCG TGACACGGCG CCCACTTACC ACCTAGGCGC TTGGTGGCC CTTCAGAAG Pro	1531
CCCTAAGTAC GGTACTTAT GCGGTAGAC ATTTATGTC ACTTATTAAG CCGCTGGCAC	1591
GGCCCTGCGT AGCAGCACCA GCGGCCCCA CCCCTGCTCG CCCCTATCGC TCCAGCCAAG	1651
GCGAAGAAGC ACGAACGAAT GTCGAGAGGG GGTGAAGACA TTCTCAACT TCTCGGCCGG	1711
AGTTTGCGTG AGATCGCGGT ATTAAATCTG TGAAGAAAA CAAACAAAA CAAAAAAA	1771
AAAAAAAA AA	1783

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 428 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg Gly Gln Leu Arg Gly
 1 5 10 15

Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu Leu Val Leu Leu Gly
 20 25 30
 Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg Cys Asp Cys Ala Gly
 35 40 45
 Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys Arg Gly Cys Pro Ala
 50 55 60
 Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro Cys Gly Asn Ser Thr
 65 70 75 80
 Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala Trp Glu Asn His His
 85 90 95
 Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp Glu Gln Ala Ser Gln
 100 105 110
 Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp Thr Arg Cys Gly Cys
 115 120 125
 Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser Gln Cys Val Ser Ser
 130 135 140
 Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys Gly Ala Leu His Arg
 145 150 155 160
 His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr Asp Cys Gly Thr Cys
 165 170 175
 Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys Val Ser Cys Pro Thr
 180 185 190
 Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala Ala Val Cys Gly Trp
 195 200 205
 Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala Gly Leu Val Val Pro
 210 215 220
 Leu Leu Leu Gly Gly Thr Leu Asp Leu His Ile Pro Pro Leu Leu Ala
 225 230 235 240
 His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly Met Glu Ala Leu Asn
 245 250 255
 Pro Pro Pro Gly Thr His Leu Ser Pro Leu Asp Ser Ala His Thr Leu
 260 265 270
 Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys Thr Val Gln Leu Val
 275 280 285
 Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr Gln Glu Ala Leu Cys
 290 295 300
 Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro Ser Arg Ala Leu Gly
 305 310 315 320
 Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser Pro Ala Gly Ser Pro
 325 330 335
 Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr Asp Val Met Asp Ala
 340 345 350
 Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg Thr Leu Gly Leu Arg
 355 360 365
 Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile Gly Arg Phe Arg Asp
 370 375 380

Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln Gln Gln Pro Ala Gly
 385 390 395 400
 Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met Gly Leu Asp Gly Cys
 405 410 415
 Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly Pro
 420 425

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 333 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
 1 5 10 15
 Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
 20 25 30
 Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Val Glu Thr Asn Leu
 35 40 45
 Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro Pro
 50 55 60
 Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro Asp
 65 70 75 80
 Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His Phe
 85 90 95
 Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Phe Gly His Gly Leu
 100 105 110
 Glu Val Glu Ile Asn Cys Thr Arg Thr Asn Thr Lys Cys Arg Cys Lys
 115 120 125
 Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp Pro Cys
 130 135 140
 Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr Ser Asn
 145 150 155 160
 Thr Lys Cys Lys Glu Glu Gly Ser Arg Ser Asn Leu Gly Trp Leu Cys
 165 170 175
 Leu Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg Lys Glu
 180 185 190
 Val Cys Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly Ser His
 195 200 205
 Glu Ser Pro Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu Ser Asp
 210 215 220
 Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr Leu
 225 230 235 240

Ser Gln Val Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu Ala Lys
 145 210 265
 Ile Asp Glu Ile Asp Asn Asp Asn Val Gln Asp Thr Ala Glu Gln Lys
 160 265 270
 Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly Lys Lys Glu Ala
 175 280 285
 Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu
 190 295 300
 Ala Glu Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser
 305 310 315 320
 Glu Asn Ser Asn Phe Arg Asn Glu Ile Gln Ser Leu Val
 325 330

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 353 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Asx Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
 1 10 15
 Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
 20 25 30
 His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
 35 40 45
 Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
 50 55 60
 Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
 65 70 75 80
 Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
 85 90 95
 Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
 100 105 110
 Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
 115 120 125
 Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
 130 135 140
 Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
 145 150 155 160
 Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
 165 170 175
 Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
 180 185 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
 195 200 205
 Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
 210 215 220
 Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
 225 230 235 240
 Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
 245 250 255
 Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
 260 265 270
 Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val
 275 280 285
 Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys Pro
 290 295 300
 Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly Ala
 305 310 315 320
 Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn Pro
 325 330 335
 Leu Gln Lys Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp Thr Asp
 340 345 350
 Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro Leu Arg
 355 360 365
 Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu Ile Asp
 370 375 380
 Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr Ser
 385 390 395 400
 Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr Leu
 405 410 415
 Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu
 420 425 430
 Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro Pro Ala
 435 440 445
 Pro Ser Leu Leu Arg
 450

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCCCATGGG GCGCCGCGG CAG

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGAAGCTTC TAGGACCCAG AACATCTGCC

30

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCGGATCCA TCGAGGAGAC GCAGCAG

27

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCGGATCCT CACCGGCGGC GCTGCA

26

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCGGATCCC AGCCCCAGGT GTGACTGTTC CGGTGACTTC CACAAGAAG

49

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCGAGATCTA GTCTGGACCC AGAACATCTG CCTCC

35

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCGGATCCA TGGAGGAGAC GAGCAG

27

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCGTCTAGAT CAAGCGTAGT CTGGAGCTC GTATCGGTAC GGGCCGCGCT GCA

53

1 WHAT IS CLAIMED IS:

2
3 1. An isolated nucleic acid molecule comprising a
4 polynucleotide having at least 70% identity to a member
5 selected from the group consisting of:

- 6 (a) a polynucleotide encoding a polypeptide comprising
7 amino acid 1 to amino acid 428 set forth in SEQ ID NO:2; and
8 (b) a polynucleotide which is complementary to the
9 polynucleotide of (a).

10
11 2. The nucleic acid molecule of claim 1 wherein the
12 polynucleotide is DNA.

13
14 3. The nucleic acid molecule of claim 1 wherein the
15 polynucleotide is RNA.

16
17 4. The nucleic acid molecule of claim 1 wherein the
18 polynucleotide is genomic DNA.

19
20 5. The nucleic acid molecule of claim 2 which encodes a
21 polypeptide comprising amino acid 1 to 428 of SEQ ID NO:2.

22
23 6. An isolated nucleic acid molecule comprising a
24 polynucleotide having at least 70% identity to a member
25 selected from the group consisting of:

- 26 (a) a polynucleotide which encodes a mature polypeptide
27 having the amino acid sequence expressed by the human cDNA
28 contained in ATCC Deposit No. 97456; and
29 (b) a polynucleotide which is complementary to the
30 polynucleotide of (a).

31
32 7. The nucleic acid molecule of claim 1 comprising the
33 sequence as set forth in SEQ ID NO:1 from nucleotide 198 to
34 nucleotide 1479.
35

1 8. The nucleic acid molecule of claim 1 comprising the
2 sequence as set forth in SEQ ID NO:1 from nucleotide 1 to
3 1780.

4
5 9. A vector comprising the nucleic acid molecule of claim
6 1.

7
8 10. A host cell comprising the vector of Claim 9.
9

10 11. A process for producing a polypeptide comprising:
11 expressing from the host cell of Claim 10 the polypeptide
12 encoded by said DNA.
13

14 12. A process for producing a cell comprising:
15 genetically engineering the cell with the vector of Claim 9
16 to thereby express the polypeptide encoded by the DNA
17 contained in the vector.
18

19 13. An isolated nucleic acid molecule comprising a
20 polynucleotide encoding a DDCR polypeptide having the amino
21 acid sequence shown in SEQ ID NO 2, or a fragment of said
22 polypeptide.
23

24 14. An isolated nucleic acid molecule having the
25 nucleotide sequence shown in SEQ ID NO 1, or a fragment
26 thereof. -
27

28 15. The isolated nucleic acid molecule of claim 14,
29 wherein said fragment comprises an open reading frame whose
30 initiation codon is at position 198-200 of the nucleotide
31 sequence shown in SEQ ID NO 1.
32

33 16. The isolated nucleic acid molecule of claim 14,
34 wherein said fragment comprises a polynucleotide encoding the
35 DDCR ligand binding domain.

1 17. The isolated nucleic acid molecule of claim 14,
2 wherein said fragment comprises a polynucleotide encoding the
3 DDCR transmembrane domain.

4
5 18. The isolated nucleic acid molecule of claim 14,
6 wherein said fragment comprises a polynucleotide encoding the
7 DDCR intracellular domain.

8
9 19. The isolated nucleic acid molecule of claim 14,
10 wherein said fragment comprises a polynucleotide encoding the
11 DDCR death domain.

12
13 20. An isolated polypeptide selected from the group
14 consisting of:

15 (a) a polypeptide having the amino acid sequence set
16 forth in SEQ ID NO:2; and

17 (b) a polypeptide which is at least 70% identical to the
18 polypeptide of (a).

19
20 21. An isolated polypeptide having the amino acid
21 sequence shown in SEQ ID NO 2, or a fragment thereof.

22
23 22. The isolated polypeptide of claim 21, wherein said
24 fragment comprises the DDCR ligand binding domain.

25
26 23. The isolated polypeptide of claim 21, wherein said
27 fragment comprises the DDCR transmembrane domain.

28
29 24. The isolated polypeptide of claim 21, wherein said
30 fragment comprises the DDCR intracellular domain.

31
32 25. The isolated polypeptide of claim 21, wherein said
33 fragment comprises the DDCR death domain.

1 26. A method for enhancing apoptosis induced by a TNF-
2 family ligand comprising administering to a cell which
3 expresses the DDCR polypeptide an effective amount of an
4 agonist capable of increasing DDCR mediated signaling.
5

6 27. The method of claim 26, wherein DDCR mediated
7 signaling is increased to treat a disease exhibiting
8 decreased apoptosis.
9

10 28. The method of claim 27, wherein said disease is
11 selected from cancer, an autoimmune disorder, viral
12 infection, or graft v. host disease.
13

14 29. The method of claims 28, wherein said cancer is
15 selected from follicular lymphomas, carcinomas with p53
16 mutations, or hormone-dependent tumors.
17

18 30. The method of claim 29, wherein said hormone-
19 dependent tumors are selected from breast cancer, prostate
20 cancer, or ovarian cancer.
21

22 31. The method of claim 28, wherein said autoimmune
23 disorder is selected from systemic lupus erythematosus or
24 immune-related glomerulonephritis.
25

26 32. The method of claim 28, wherein said viral infection
27 is selected from herpesvirus, poxvirus, or adenovirus
28 infection.
29

30 33. A method for inhibiting apoptosis induced by a TNF-
31 family ligand comprising administering to a cell which
32 expresses the DDCR polypeptide an effective amount of an
33 antagonist capable of decreasing DDCR mediated signaling.
34

1- 34. The method of claim 33, wherein DDCR mediated
2 signaling is decreased to treat a disease exhibiting
3 increased apoptosis.
4

5 35. The method of claim 34, wherein said disease is
6 selected from AIDS, neurodegenerative disorders,
7 myelodysplastic syndromes, ischemic injury, toxin-induced
8 liver disease, septic shock, cachexia, or anorexia.
9

10 36. The method of claim 35, wherein said
11 neurodegenerative disorder is selected from Alzheimer's
12 disease, Parkinson's disease, Amyotrophic lateral sclerosis,
13 Retinitis pigmentosa, or Cerebellar degeneration.
14

15 37. The method of claim 35, wherein said myelodysplastic
16 syndrome is aplastic anemia.
17

18 38. The method of claim 35, wherein said ischemic injury
19 is caused by myocardial infarction, stroke or reperfusion.
20

21 39. The method of claim 35, wherein said toxin-induced
22 liver disease is caused by alcohol.
23

24 40. The method of claim 26, wherein said agonist is
25 selected from TNF family ligand peptide fragments,
26 transforming growth factor β peptide fragments,
27 neurotransmitters, tumor suppressors, cytolytic T cells,
28 antimetabolites, chemotherapeutic drugs, anti-DDCR polyclonal
29 antibodies, or anti-DDCR monoclonal antibodies.
30

31 41. The method of claim 40, wherein said neurotransmitter
32 is selected from glutamate, dopamine, or N-methyl-D-
33 aspartate.
34

1 42. The method of claim 40, wherein said chemotherapeutic
2 drug is selected from cisplatin, doxorubicin, bleomycin,
3 cytosine arabinoside, nitrogen mustard, methotrexate, or
4 vincristine.

5
6 43. The method of claim 33, wherein said antagonist is
7 selected from CD40 ligand neutral amino acids, zinc,
8 estrogen, androgens, calpain inhibitors, cysteine protease
9 inhibitors, tumor promoters, anti-DDCR polyclonal antibodies,
10 anti-DDCR monoclonal antibodies, or a soluble polypeptide
11 comprising the DDCR extracellular domain.
12

13 44. The method of claim 43, wherein said tumor promoter
14 is selected from PMA, Phenobarbital, or α -
15 Hexachlorocyclohexane.
16

17 45. A screening method for determining whether a
18 candidate agonist is capable of enhancing a cellular response
19 to a TNF-family ligand, comprising:

20 (a) contacting cells which express the DDCR polypeptide
21 with a candidate agonist and a TNF-family ligand;

22 (b) assaying a cellular response;

23 (c) comparing said cellular response to a standard
24 cellular response, said standard being assayed when contact
25 is made with the ligand in absence of the candidate agonist;
26 whereby,

27 an increased cellular response over the standard indicates
28 that the candidate agonist is a potentiator of DDCR-mediated
29 signaling.
30

31 46. A screening method for determining whether a
32 candidate antagonist is capable of inhibiting a cellular
33 response to a TNF-family ligand, comprising:

34 (a) contacting cells which express the DDCR polypeptide
35 with a candidate antagonist and a TNF-family ligand;

1 (b) assaying a cellular response;
2 (c) comparing said cellular response to a standard
3 cellular response, said standard being assayed when contact
4 is made with the ligand in absence of the candidate
5 antagonist; whereby,
6 a decreased cellular response over said standard indicates
7 that the candidate antagonist is an inhibitor of DDCR-
8 mediated signaling.

10 47. The method of claim 45, wherein said cell is
11 contacted with an endogenous TNF-family ligand.
12

13 48. The method of claim 46, wherein said cell is
14 contacted with an endogenous TNF-family ligand.
15

16 49. The method of claim 45, wherein said cell is
17 contacted with an exogenously administered TNF-family ligand.
18

19 50. The method of claim 46, wherein said cell is
20 contacted with an exogenously administered TNF-family ligand.
21

ABSTRACT OF THE DISCLOSURE

The invention concerns a novel member of the tumor necrosis factor (TNF) receptor family. In particular, isolated nucleic acid molecules are provided encoding death domain containing (DDCR) polypeptides. DDCR polypeptides are also provided as are screening methods for identifying agonist and antagonist capable of potentiating or inhibiting DDCR-mediated signaling. The invention further concerns therapeutic methods for treating diseases associated with dysregulation of apoptosis.

60/013285

```
1210      1230      1250
CCCCAGCCATGATGCTGCAGCCGGGCGGCGAGCTCTACGACGTGATGGACGGGTCCCAG
P A M H L Q P G P Q L Y D V M D A V P A
1270      1290      1310
CGCGCGCGCTGGAAGGAGTTCTGTCGCCACGCTGGGGCTGGCGGAGGCAGATCGAAGCCG
R R W K E P V R T L G L R E A E I E A V
1330      1350      1370
TGGAGGTGGAGATCGGCGGCTTCCGAGACCAGCAGTACGAGATGCTCAAGCGCTGGCGCC
E V E I G R F R D Q Q Y E M L K R W R Q
1390      1410      1430
AGCAGCAGCCCGCGGCGCTCGGAGCGGTTTACGCGGCGCTGGAGCGCATGGGGCTGGAGG
Q Q P A G L G A V Y A A L E R H G L D G
1450      1470      1490
GCTGCGTGGAAAGACTTGGCGAGCTGCTGCAGCGCGGCGCGGTGACACGGCGCCCACTTC
C V E D L R S E L Q R G P
1510      1530      1550
CACCTAGGCGCTCTGCTGCCCGCTTSCAGAAGCCCTAAGTACGGTTACTTATGCGGTAGA
1570      1590      1610
CATTTTATGTCACCTATTAAAGCGGCTGGCACGGCGCTGCGTAGCAGCAGCAGCGCGCCCC
1630      1650      1670
ACCCCTGCTCGGCCCTATCGCTCCAGCCAAAGGCGAAGCAACGAACGATGTGGAGAGG
1690      1710      1730
GGGTCAAGACATTTCTCAACTTCTCGGCGCGGAGTTTGGCTGAGATCGCGGTATTAATCT
1750      1770
GTGAAAGAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAAC
```

FIGURE 1. (CONT.)

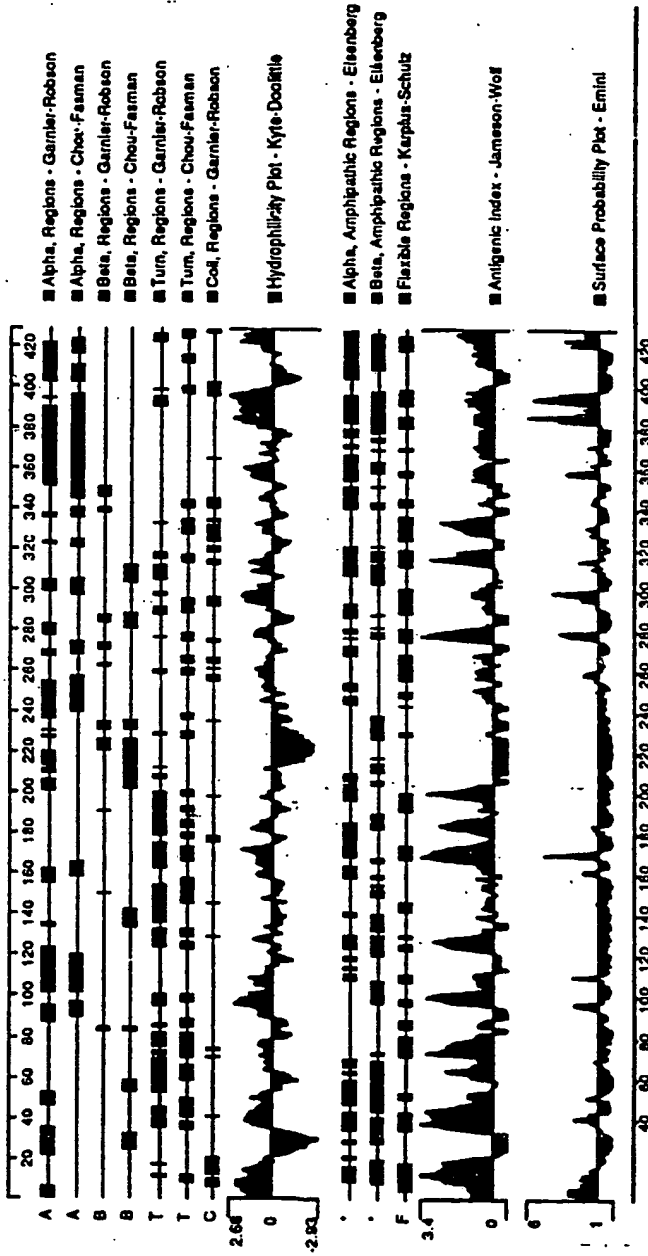


FIGURE 3

60/013285



60/013285

EXPRESS MAIL CERTIFICATE

"Express Mail" Number _____ Date of Deposit _____
I hereby certify that this application is being deposited with the United States Postal Service
"Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above
and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

(Typed or Printed Name of Person Mailing Application)

(Signature of Person Mailing Application)

U.S. PATENT AND TRADEMARK OFFICE
PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a Provisional Application for Patent under CFR 1.53 (b) (2).

Docket No.	PF267FP	Type a plus sign (+) inside this box →	+
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INVENTOR (S) / APPLICANTS (S)

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TITLE OF THE INVENTION (280 characters max.)

Death Domain Containing Receptor

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ENCLOSED APPLICATION PARTS (check all that apply)

<input checked="" type="checkbox"/> Specification	Number of Pages -74-	<input type="checkbox"/> Small Entity Statement
<input checked="" type="checkbox"/> Drawings	Number of Sheets -4-	<input checked="" type="checkbox"/> Other Specify -Sequence Listing+ diskette

METHOD OF PAYMENT (check one)

<input type="checkbox"/> A check or money order is enclosed to cover the Provisional filing fee
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 08-3425

PROVISIONAL FILING FEE AMOUNT (\$)	\$130.00
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The invention was made by an agency of the U.S. Government or under a contract with an agency of the United States Government

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully Submitted,
Signature Robert H. Benson Date: March 12, 1996Typed or Printed Name Robert H. Benson Registration No. (if appropriate) 30,446☒ Additional inventors are being named on separately numbered sheet attached hereto.

PROVISIONAL APPLICATION FILING ONLY

WPI P Open Form/ KR PTO/ Prov-HGS.doc

ANNEX TO
PROVISIONAL APPLICATION COVER SHEET

Docket No. PF267pp Title: Death Domain Containing Receptor

■ ADDITIONAL INVENTORS

Last Name	First Name	Middle Initial	Residence (City and Either State or Foreign Country)
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Death Domain Containing Receptors

Background of the Invention

Field of the Invention

5 The present invention relates to a novel member of the tumor necrosis factor family of receptors. More specifically, isolated nucleic acid molecules are provided encoding human Death Domain Containing Receptors (DR3 and DR3-V1). Death Domain Containing Receptor polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The
10 invention further relates to screening methods for identifying agonists and antagonists of DR3 activity.

Related Art

 Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many
15 cytokines act through receptors by engaging the receptor and producing an intracellular response.

 For example, tumor necrosis factors (TNF) alpha and beta are cytokines which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory
20 disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

 Among the ligands there are included TNF- α , lymphotoxin- α (LT- α , also
25 known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40L, CD27L, CD30L, 4-1BBL, OX40L and nerve growth factor (NGF). The

TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 4-1BB, OX40, low affinity p75 and NGF-receptor (Meager, A., *Biologicals*, 22:291-295 (1994)).

5 Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., *supra*).

10 Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., *et al.*, *Nature* 356:314 (1992)), perhaps reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating
15 faulty T-cell-dependent B-cell activation (Allen, R.C. *et al.*, *Science* 259:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by faulty sensory innervation of peripheral structures (Lee, K.F. *et al.*, *Cell* 69:737 (1992)).

20 TNF and LT- α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT- α , acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT- α are involved
25 in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von Huffel, C., *Science* 264:667-668 (1994)). Mutations in the p55 Receptor cause increased susceptibility to microbial infection.

30 Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is responsible for

transducing signals for programmed cell death (Tartaglia *et al.*, *Cell* 74:845 (1993)).

Apoptosis, or programmed cell death, is a physiologic process essential to the normal development and homeostasis of multicellular organisms (H. Steller, *Science* 267, 1445-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, *Science* 267, 1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland, *et al.*, *Cell* 81, 479-482 (1995); A. Fraser, *et al.*, *Cell* 85, 781-784 (1996); S. Nagata, *et al.*, *Science* 267, 1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40, and CD30, among others (C.A. Smith, *et al.*, *Science* 248, 1019-23 (1990); M. Tewari, *et al.*, in *Modular Texts in Molecular and Cell Biology* M. Purton, Heldin, Carl, Ed. (Chapman and Hall, London, 1995). While family members are defined by the presence of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain", which is distantly related to the *Drosophila* suicide gene, reaper (P. Golstein, *et al.*, *Cell* 81, 185-6 (1995); K. White *et al.*, *Science* 264, 677-83 (1994)). This shared death domain suggests that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M. P. Boldin, *et al.*, *J. Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio *et al.*, *Cell* 85, 817-827 (1996); M.P. Boldin, *et al.*, *Cell* 85, 803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF-kB (L.A. Tartaglia, *et al.*, *Immunol Today* 13, 151-3

(1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu, *et al.*, *Cell* 81, 495-504 (1995); H. Hsu, *et al.*, *Cell* 84, 299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF-kB activation (H. Hsu, *et al.*, *Cell* 84, 299-308 (1996); H. Hsu, *et al.*, *Immunity* 4, 387-396 (1996)).

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states. In particular, there is a need to isolate and characterize novel members of the TNF receptor family.

Summary of the Invention

The present invention provides for isolated nucleic acid molecules comprising nucleic acid sequences encoding the amino acid sequences shown in FIG. 1 (SEQ ID NO:2) and FIG. 2 (SEQ ID NO:4) or the amino acid sequence encoding the cDNA clones deposited in a bacterial host as ATCC Deposit No. 97456 on March 1, 1996 and ATCC Deposit No. ____ on October 10, 1996.

The present invention also provides vectors and host cells for recombinant expression of the nucleic acid molecules described herein, as well as to methods of making such vectors and host cells and for using them for production of DR3 or DR3 Variant 1 (DR3-V1) (formerly named DDCR) polypeptides or peptides by recombinant techniques.

The invention further provides an isolated DR3 or DR3-V1 polypeptide having an amino acid sequence encoded by a polynucleotide described herein.

The present invention also provides diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR3 or DR3-V1 protein. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-

expression of DR3 or DR3-V1, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors.

5 Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. Cellular response to TNF-family ligands include not only normal physiological responses, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death-is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes. Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers, autoimmune disorders, viral infections, inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia.

10 Thus, the invention further provides a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR3 polypeptide an effective amount of an agonist capable of increasing DR3 mediated signaling. Preferably, DR3 mediated signaling is increased to treat a disease wherein decreased apoptosis is exhibited.

20 In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR3 polypeptide an effective amount of an antagonist capable of decreasing DR3 mediated signaling. Preferably, DR3 mediated signaling is decreased to treat a disease wherein increased apoptosis is exhibited.

25 Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail

30

below. Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR3 or DR3-V1 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By the invention, a cell expressing the DR3 or DR3-V1 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Brief Description of the Figures

FIG. 1A-B shows the nucleotide and deduced amino acid sequence of DR3-V1. It is predicted that amino acids 1 - 35 constitute the signal peptide, amino acids 36-212 constitute the extracellular domain, amino acids 213-235 constitute the transmembrane domain, amino acids 236-428 constitute the intracellular domain, and amino acids 353-419 the death domain.

FIG. 2 shows the nucleotide and deduced amino acid sequence of DR3. It is predicted that amino acids 1 - 24 constitute the signal peptide, amino acids 25-201 constitute the extracellular domain, amino acids 202-224 constitute the transmembrane domain, amino acids 225-417 constitute the intracellular domain, and amino acids 342-408 constitute the death domain.

FIG. 3 shows the regions of similarity between the amino acid sequences of the DR3-V1, human tumor necrosis factor receptor 1, and Fas receptor [SEQ ID NOs:5 and 6].

FIG. 4 shows an analysis of the DR3-V1 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic

regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues 1-22, 33-56, 59-82, 95-112, 122-133, 161-177, 179-190, 196-205 in Figure 1 correspond to the shown highly antigenic regions of the DR3-V1 protein.

Detailed Description of the Preferred Embodiments

The present invention provides isolated nucleic acid molecules comprising a nucleic acid sequence encoding the DR3-V1 or DR3 polypeptide whose amino acid sequence is shown in FIG. 1 [SEQ ID NO:2] and FIG. 2 [SEQ ID NO:4], respectively, or a fragment of the polypeptide. The DR3-V1 and DR3 polypeptides of the present invention shares sequence homology with human TNF RI and Fas (FIG. 4). The nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] was obtained by sequencing the HTTNB61 clone, which was deposited on March 1, 1996 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given Accession Number 97456. The deposited clone is contained in the pBluescript SK(-) plasmid (Stratagene, LaJolla, CA). The nucleotide sequence shown in FIG. 2 [SEQ ID NO:3] was obtained by sequencing a clone obtained from a HUVEC library, which was deposited on October 10, 1996 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given Accession Number _____. The deposited clone is contained in the pBluescript SK(-) plasmid (Stratagene, LaJolla, CA).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for purposes of the invention as are native or recombinant polypeptides which have been substantially purified by any suitable technique such as, for example, the single-step purification method disclosed in Smith and Johnson, *Gene* 67:31-40 (1988).

Using the information provided herein, such as the nucleic acid sequence set out in FIG. 1 or FIG. 2, a nucleic acid molecule of the present invention encoding a DR3-V1 or DR3 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as

starting material. Illustrative of the invention, the nucleic acid molecule described in FIG. 1 was discovered in a cDNA library derived from cells of a human testis tumor. Also illustrative of the invention, the nucleic acid molecule described in FIG. 2 was discovered in a human HUVEC cDNA library. In addition, the genes of the present invention have also been identified in cDNA libraries of the following tissues: fetal liver, fetal brain, tonsil and leukocyte. Furthermore, multiple forms of DR3 transcript are seen in Northern Blots and PCR reactions indicating that multiple variants of the transcript exists, possibly due to alternate splicing of the message.

The DR3-V1 (formerly called DDCR) gene contains an open reading frame encoding a protein of about 428 amino acid residues whose initiation codon is at position 198-200 of the nucleotide sequence shown in FIG. 1 [SEQ ID NO:1], with a leader sequence of about 35 amino acid residues, and a deduced molecular weight of about 47 kDa. Of known members of the TNF receptor family, the DR3-V1 polypeptide of the invention shares the greatest degree of homology with human TNF R1. The DR3-V1 polypeptide shown in FIG. 1 [SEQ ID NO:2] is about 20% identical and about 50% similar to human TNF R1.

The DR3 gene contains an open reading frame encoding a protein of about 417 amino acid residues whose initiation codon is at position 1-3 of the nucleotide sequence shown in FIG. 2 [SEQ ID NO:3], with a leader sequence of about 24 amino acid residues, and a deduced molecular weight of about 43 kDa. Of known members of the TNF receptor family, the DR3 polypeptide of the invention shares the greatest degree of homology with human TNF R1. The DR3 polypeptide shown in FIG. 2 [SEQ ID NO:3] is about 20% identical and about 50% similar to human TNF R1.

As indicated, the present invention also provides the mature form(s) of the DR3-V1 and DR3 protein of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Most mammalian cells and even insect cells cleave secreted proteins with the

same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species on the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding the mature DR3-V1 or DR3 polypeptides having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97456 and _____, respectively, and as shown in Figure 1 (SEQ ID NO:2) and Figure 2 (SEQ ID NO:4). By the mature DR3-V1 or DR3 protein having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97456 and _____, respectively, is meant the mature form(s) of the DR3-V1 or DR3 protein produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the human DNA sequence of the clone contained in the vector in the deposited host. As indicated below, the mature DR3-V1 or DR3 having the amino acid sequence encoded by the cDNA clones contained in ATCC Deposit No. 97456 and _____, respectively, may or may not differ from the predicted "mature" DR3-V1 protein shown in Figure 1 (amino acids from about 36 to about 428) or DR3 protein shown in Figure 2 (amino acids from about 24 to about 417) depending on the accuracy of the predicted cleavage site based on computer analysis.

Methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the method of McGeoch (*Virus Res.* 3:271-286 (1985)) and von Heinje (*Nucleic Acids Res.* 14:4683-4690 (1986)) can be used. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 75-80%. von Heinje, *supra*. However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the predicted amino acid sequence of the complete DR3-V1 and DR3 polypeptides of the present invention were analyzed by a

computer program ("PSORT"). (see K. Nakai and M. Kanehisa, *Genomics* 14:897-911 (1992)), which is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis by the PSORT program predicted the cleavage sites between amino acids 35 and 36 in Figure 1 (SEQ ID NO:2) and between amino acids 24 and 25 in Figure 2 (SEQ ID NO:4). Thereafter, the complete amino acid sequences were further analyzed by visual inspection, applying a simple form of the (-1,-3) rule of von Heine. von Heinje, *supra*. Thus, the leader sequence for the DR3-V1 protein is predicted to consist of amino acid residues 1- 35 in Figure 1 (SEQ ID NO:2), while the predicted mature DR3-V1 protein consists of residues 36-428. The leader sequence for the DR3 protein is predicted to consist of amino acid residues 1- 24 in Figure 2 (SEQ ID NO:4), while the predicted mature DR3 protein consists of residues 25-417.

As one of ordinary skill would appreciate, due to the possibilities of sequencing errors discussed above, as well as the variability of cleavage sites for leaders in different known proteins, the actual DR3-V1 polypeptide encoded by the deposited cDNA comprises about 428 amino acids, but may be anywhere in the range of 410-440 amino acids; and the actual leader sequence of this protein is about 35 amino acids, but may be anywhere in the range of about 25 to about 45 amino acids. The actual DR3 polypeptide encoded by the deposited cDNA comprises about 417 amino acids, but may be anywhere in the range of 400-430 amino acids; and the actual leader sequence of this protein is about 24 amino acids, but may be anywhere in the range of about 14 to about 34 amino acids.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DR3-V1 DNA molecules comprising an open reading frame (ORF) shown in FIG. 1 [SEQ ID NO:1] and further include DNA molecules which comprise a sequence substantially different than all or part of the ORF whose initiation codon is at position 198-200 of the nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] but which, due to the degeneracy of the genetic code, still encode the DR3-V1 polypeptide or a fragment thereof. Isolated nucleic acid molecules of the present invention also include DR3 DNA molecules comprising an open reading frame (ORF) shown in FIG. 2 [SEQ ID NO:3] and further include DNA molecules which comprise a sequence substantially different than all or part of the ORF whose initiation codon is at position 1-3 of the nucleotide sequence shown in FIG. 2 [SEQ ID NO:3] but which, due to the degeneracy of the genetic code, still encode the DR3 polypeptide or a fragment thereof. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In another aspect, the invention provides isolated nucleic acid molecules encoding the DR3-V1 polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97456 on March 1, 1996. The invention provides isolated nucleic acid molecules encoding the DR3 polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. _____ on October 10, 1996. Preferably, these nucleic acid molecules will encode the

mature polypeptide encoded by the above-described deposited cDNA clone. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in Figure 1 (SEQ ID NO:1) or FIG. 2 (SEQ ID NO:3) or the nucleotide sequence of the DR3-V1 or DR3 cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated DNA molecules and fragments thereof are useful as DNA probes for gene mapping by *in situ* hybridiz DR3-V1 or DR3 gene in human tissue (including testis tumor tissue) by Northern blot analysis.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By fragments of an isolated DNA molecule having the nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] or FIG. 2 [SEQ ID NO:3] are intended DNA fragments at least 20 bp, and more preferably at least 30 bp in length which are useful as DNA probes as discussed above. of course larger DNA fragments 50-1500 bp in length are also useful as DNA probes according to the present invention as are DNA fragments corresponding to most, if not all, of the nucleotide sequence shown in FIG. 1 [SEQ ID NO:1] or FIG. 2 [SEQ ID NO:3]. By a fragment at least 20 bp in length, for example, is intended fragments which include 20 or more bases from the nucleotide sequence in FIG. 1 [SEQ ID NO:1] or FIG. 2 [SEQ ID NO:3].

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising the DR3 extracellular domain (amino acid residues from about 36 to about 212 in FIG. 1 [SEQ ID NO:2]); a polypeptide comprising the DR3 transmembrane domain (amino acid residues from about 213 to about 235 in FIG. 1 [SEQ ID NO:2]; a polypeptide comprising the DR3 intracellular domain (amino acid residues from about 214 to about 428 in FIG. 1 [SEQ ID NO:2] ; and a polypeptide comprising the DR3 death domain (amino acid residues from about 353 to about 419 in FIG. 1 [SEQ ID NO:2]). Since the location of these domains have been predicted by computer graphics, one of ordinary skill would appreciate that the amino acid residues constituting

these domains may vary slightly (e.g., by about 1 to 15 residues) depending on the criteria used to define the domain.

Preferred nucleic acid fragments of the present invention further include nucleic acid molecules encoding epitope-bearing portions of the DR3-V1 protein.

5 In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 1 to about 22 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 33 to about 56 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 59 to about 82 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 95 to about 112 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 122 to about 133 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 161 to about 177 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 179 to about 190 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 196 to about 205 in Figure 1 (SEQ ID NO:2). The inventors have determined that the above polypeptide fragments are antigenic regions of the DR3-V1 protein. Methods for determining other such epitope-bearing portions of the DR3-V1 protein are described in detail below.

20 Preferred nucleic acid fragments of the present invention also include nucleic acid molecules encoding epitope-bearing portions of the DR3 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding the corresponding regions to those epitope-bearing regions of the DR3-V1 protein disclosed above. Methods for determining other such epitope-bearing portions of the DR3 protein are described in detail below.

25 In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clones contained in ATCC Deposit 97456 or ATCC Deposit _____. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50%

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formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

5 By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed
10 above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNA or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1) or Figure 2 (SEQ ID NO:3).

15 Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of the DR3-V1 cDNA shown in Figure 1 (SEQ ID NO:1)), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any
20 nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode the DR3-V1 or DR3 polypeptide may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the
25 mature polypeptide and additional sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3'
30 sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals,

for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37:767 (1984), for instance.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode for fragments, analogs or derivatives of the DR3-V1 or DR3 polypeptide. Variants may occur naturally, such as an allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions which may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

Further embodiments of the invention include isolated nucleic acid molecules that are at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to (a) a nucleotide sequence encoding the full-length DR3-V1 polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence; (b) nucleotide sequence encoding the full-length DR3 polypeptide having the complete amino acid

sequence in Figure 2 (SEQ ID NO:4), including the predicted leader sequence;
(c) a nucleotide sequence encoding the mature DR3-V1 polypeptide (full-length
polypeptide with the leader removed) having the amino acid sequence at positions
about 36 to about 428 in Figure 1 (SEQ ID NO:2); (d) a nucleotide sequence
5 encoding the full-length DR3-V1 polypeptide having the complete amino acid
sequence including the leader encoded by the cDNA clone contained in ATCC
Deposit No. 97456; (e) a nucleotide sequence encoding the full-length DR3
polypeptide having the complete amino acid sequence including the leader
encoded by the cDNA clone contained in ATCC Deposit No. _____; (f) a
10 nucleotide sequence encoding the mature DR3-V1 polypeptide having the amino
acid sequence encoded by the cDNA clone contained in ATCC Deposit No.
97456; (g) a nucleotide sequence encoding the mature DR3-V1 polypeptide
having the amino acid sequence encoded by the cDNA clone contained in ATCC
Deposit No. _____; (h) a nucleotide sequence that encodes the DR3 extracellular
15 domain, (i) a nucleotide sequence that encodes the DR3 transmembrane domain,
(j) a nucleotide sequence that encodes the DR3 intracellular domain, and (k) a
nucleotide sequence that encodes the DR3 death domain; or (l) a nucleotide
sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d),
(e), (f), (g), (h), (i), (j) or (k) above.

20 By a polynucleotide having a nucleotide sequence at least, for example,
95% "identical" to a reference nucleotide sequence encoding a DR3-V1 or DR3
polypeptide is intended that the nucleotide sequence of the polynucleotide is
identical to the reference sequence except that the polynucleotide sequence may
include up to five point mutations per each 100 nucleotides of the reference
25 nucleotide sequence encoding the DR3-V1 or DR3 polypeptide. In other words,
to obtain a polynucleotide having a nucleotide sequence at least 95% identical to
a reference nucleotide sequence, up to 5% of the nucleotides in the reference
sequence may be deleted or substituted with another nucleotide, or a number of
nucleotides up to 5% of the total nucleotides in the reference sequence may be
30 inserted into the reference sequence. These mutations of the reference sequence
may occur at the 5' or 3' terminal positions of the reference nucleotide sequence

or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figure 1, Figure 2 or to the nucleotide sequences of the deposited cDNA clones can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1), Figure 2 (SEQ ID NO:3) or to the nucleic acid sequence of the deposited cDNAs, irrespective of whether they encode a polypeptide having DR3 activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having DR3 activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having DDCR activity include, *inter alia*, (1) isolating the DR3-V1 or DR3 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the DR3-V1 or DR3 gene, as described in Verma *et al.*, *Human*

Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting DR3-V1 or DR3 mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1), Figure 2 (SEQ ID NO:3) or to the nucleic acid sequence of the deposited cDNAs which do, in fact, encode a polypeptide having DR3 protein activity. By "a polypeptide having DR3 activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the DR3 protein of the invention (either the full-length protein or, preferably, the mature protein), as measured in a particular biological assay. For example, DR3 protein activity can be measured using the cell death assays performed essentially as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)) and as set forth in Example 7, below. In MCF7 cells, plasmids encoding full-length DR3 or a candidate death domain containing receptors are co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DR3 will exhibit apoptotic morphology as assessed by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin, *et al.*, *Cell* 85, 803-815 (1996); M. Tewari, *et al.*, *J Biol Chem* 270, 3255-60 (1995)), DR3-induced apoptosis is blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk. In addition, apoptosis induced by DR3 is also blocked by dominant negative versions of FADD (FADD-DN) or FLICE (FLICE-DN/MACHa1C360S).

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited cDNA or the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or Figure 2 (SEQ ID NO:3) will

encode a polypeptide "having DR3 protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having DR3 protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Polynucleotide assays

This invention is also related to the use of the DR3-V1 or DR3 polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of DR3-V1 or DR3 associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of DR3-V1 or DR3 or a soluble form thereof, such as, for example, tumors or autoimmune disease.

Individuals carrying mutations in the DR3-V1 or DR3 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. (Saiki *et al.*, *Nature* 324:163-166 (1986)). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid

encoding DR3-V1 or DR3 can be used to identify and analyze DR3-V1 or DR3 expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled DR3-V1 or DR3 RNA or alternatively, radiolabeled DR3-V1 or DR3 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science* 230:1242 (1985)).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA

sequencing or the use of restriction enzymes, (e.g., restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

5 ***Chromosome assays***

10 The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

15 In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of a DR3-V1 or a DR3 gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA the is used for *in situ* chromosome mapping using well known techniques for this purpose.

20 In addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

25 Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60. For a review of this technique, see Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian*

Inheritance in Man, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes)).

5 Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Vectors and Host Cells

10 The present invention also relates to vectors which include DNA molecules of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

15 Host cells can be genetically engineered to incorporate nucleic acid molecules and express polypeptides of the present invention. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention.

20 In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally
25 will occur only in complementing host cells.

 Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-

acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

5 A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, from bacteriophage, from
10 yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

15 The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s)), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. In general, expression constructs will contain sites for
20 transcription, initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

25 In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may
30 contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic

trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

5 The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast
10 cells; insect cells such as *Drosophila* S2 and *Spodoptera Sf9* cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Hosts for of a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention.

15 Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1
20 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors available to those of skill in the art.

25 Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

30 The present invention also relates to host cells containing the above-described constructs discussed above. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as

5 Davis *et al.*, *Basic Methods in Molecular Biology* (1986).

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the

10 polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability

15 and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another

20 human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous

25 manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL-5- has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, Vol. 8:52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, Vol. 270, No. 16:9459-9471 (1995).

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The DR3 and DR3-V1 polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

DR3-V1 or DR3 polynucleotides and polypeptides may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties of DR3. Among these are applications in treatment of tumors, resistance to parasites, bacteria and viruses, to induce proliferation of T-cells, endothelial cells and certain hematopoietic cells, to treat restenosis, graft vs. host disease, to regulate anti-viral responses and to prevent certain autoimmune diseases after stimulation of DR3 by an agonist. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are discussed further below.

DR3 Polypeptides and Fragments

The invention further provides an isolated DR3-V1 or DR3 polypeptide having the amino acid sequence shown in FIG. 1 [SEQ ID NO:2] and FIG. 2[SEQ ID NO:4], respectively, or a fragment thereof. It will be recognized in the art that some amino acid sequence of DR3-V1 or DR3 can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Such areas will usually comprise residues which make up the ligand binding site or the death domain, or which form tertiary structures which affect these domains.

Thus, the invention further includes variations of the DR3-V1 or DR3 protein which show substantial DR3 protein activity or which include regions of DR3-V1 or DR3 such as the protein fragments discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U. *et al.*, *Science* 247:1306-1310 (1990).

Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the DR3-V1 or DR3 protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al.*, *Clin Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36:838-845 (1987); Cleland *et al.* *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al.*, *Nature* 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF- α to only one of the two known types of TNF receptors. Thus, the DR3-V1 or DR3 receptor of the present

invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

Amino acids in the DR3-V1 or DR3 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization,

nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al. Science* 255:306-312 (1992)).

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly
5 produced version of the DR3-V1 or DR3 polypeptide is substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988).

The polypeptides of the present invention also include the polypeptide encoded by the deposited cDNA including the leader, the mature polypeptide encoded by the deposited the cDNA minus the leader (i.e., the mature protein),
10 the polypeptide of Figure 1 (SEQ ID NO:2) or Figure 2 (SEQ ID NO:4) including the leader, the polypeptide of Figure 1 (SEQ ID NO:2) or Figure 2 (SEQ ID NO:4) minus the leader, the extracellular domain, the transmembrane domain, the intracellular domain, soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain as
15 well as polypeptides which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide encoded by the deposited cDNA clones, to the polypeptide of Figure 1 (SEQ ID NO:2) or Figure 2 (SEQ ID NO:4), and also include portions of such polypeptides with at least 30 amino acids and more
20 preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a DR3-V1 or DR3 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may
25 include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the DR3-V1 or DR3 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a
30 number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the

reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

5 As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figure 1 (SEQ ID NO:2), or Figure 2 (SEQ ID NO:4) or to the amino acid sequence encoded by deposited cDNA clones can be determined conventionally using known computer programs such the Bestfit program
10 (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the
15 percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

 The present inventors have discovered that the DR3-V1 polypeptide is a 428 residue protein exhibiting three main structural domains. First, the ligand
20 binding domain was identified within residues from about 36 to about 212 in FIG. 1 [SEQ ID NO:2]. Second, the transmembrane domain was identified within residues from about 213 to about 235 in FIG. 1 [SEQ ID NO:2]. Third, the intracellular domain was identified within residues from about 236 to about 428 in FIG. 1 [SEQ ID NO:2]. Importantly, the intracellular domain includes a
25 death domain at residues from about 353 to about 419. Further preferred fragments of the polypeptide shown in FIG. 1 [SEQ ID NO:2] include the mature protein from residues about 36 to about 428 and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain.

30 The present inventors have also discovered that the DR3 polypeptide is a 417 residue protein exhibiting three main structural domains. First, the ligand

binding domain was identified within residues from about 25 to about 201 in FIG. 2 [SEQ ID NO:4]. Second, the transmembrane domain was identified within residues from about 202 to about 224 in FIG. 2 [SEQ ID NO:4]. Third, the intracellular domain was identified within residues from about 225 to about 417 in FIG. 2 [SEQ ID NO:4]. Importantly, the intracellular domain includes a death domain at residues from about 342 to about 408. Further preferred fragments of the polypeptide shown in FIG. 2 [SEQ ID NO:4] include the mature protein from residues about 25 to about 417 and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain. As one of skill in the art will recognize, the full length polypeptides encoded by the DR3-V1 and DR3 cDNA differ only in the amino acid sequence of the leader peptide. The first 24 amino acids of the polypeptide shown in Figure 1 are replaced by the first 13 amino acids shown in Figure 2 but the rest of the amino acid sequence is the same. Thus, both the DR3-V1 cDNA and DR3 cDNA encode an identical mature protein having the same biological activity.

Thus, the invention further provides DR3-V1 or DR3 polypeptides encoded by the deposited cDNA clones including the leader and DR3-V1 or DR3 polypeptide fragments selected from the mature protein, the extracellular domain, the transmembrane domain, the intracellular domain, and the death domain.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide described herein. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A. (1983) Antibodies that react with predetermined sites on proteins. *Science* 219:660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al.*, *Cell* 37:767-778 (1984) at 777.

Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between at least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate DR3-specific antibodies include: a polypeptide comprising amino acid residues from about 1 to about 22 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 33 to about 56 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 59 to about 82 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 95 to about 112 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 122 to about 133 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 161 to about 177 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 179 to about 190 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 196 to about 205 in

Figure 1 (SEQ ID NO:2). In addition, antigenic polypeptides or peptides include polypeptides comprising the amino acid residues that are the corresponding residues to those polypeptides of DR3-V1 disclosed above. As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the DR3-V1 and DR3 protein.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Houghten, R.A., "General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids," *Proc. Natl. Acad. Sci. USA* 82:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

As one of skill in the art will appreciate, DR3-V1 or DR3 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al.*, *Nature* 331:84-86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric DR3-V1 or DR3 protein or protein fragment alone (Fountoulakis *et al.*, *J Biochem* 270:3958-3964 (1995)).

Polypeptide assays

The present invention also relates to diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR3-V1 or DR3 protein, or the soluble form thereof, in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the

invention for detecting over-expression of DR3-V1 or DR3, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors, for example. Assay techniques that can be used to determine levels of a protein, such as an DR3 protein of the present invention, or a soluble form thereof, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

Assaying DR3-V1 or DR3 protein levels in a biological sample can occur using any art-known method. Preferred for assaying DR3-V1 or DR3 protein levels in a biological sample are antibody-based techniques. For example, DR3-V1 or DR3 protein expression in tissues can be studied with classical immunohistological methods. (Jalkanen, M., *et al.*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., *et al.*, *J. Cell. Biol.* 105:3087-3096 (1987)).

Other antibody-based methods useful for detecting DR3-V1 or DR3 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as glucose oxidase, radioisotopes, such as iodine (^{125}I , ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^3H), indium (^{112}In), and technetium ($^{99\text{m}}\text{Tc}$), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Therapeutics

The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol.* 51:597-609 (1986), Cold Spring Harbor; Beutler, B., and Cerami, A., *Annu. Rev. Biochem.* 57:505-518 (1988); Old, L.J., *Sci. Am.* 258:59-75 (1988); Fiers, W., *FEBS Lett.* 285:199-224 (1991)). The TNF-family ligands induce such various

cellular responses by binding to TNF-family receptors, including the DR3-V1 or DR3 of the present invention. Cells which express the DR3-V1 or DR3 polypeptide and are believed to have a potent cellular response to DR3-V1 or DR3 ligands include lymphocytes, fibroblasts, macrophages, synovial cells, activated T-cells, lymphoblasts and epithelial cells. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death-is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (Ameisen, J.C., *AXDS* 8:1197-1213 (1994); Krammer, P.H. *et al.*, *Curr. Opin. Immunol.* 6:279-289 (1994)).

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, such as breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), information graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); myelodysplastic syndromes (such as aplastic anemia), ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Thus, in one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR3-V1 or DR3 polypeptide an

effective amount of DR3-V1 or DR3 ligand, analog or an agonist capable of increasing DR3-V1 or DR3 mediated signaling. Preferably, DR3-V1 or DR3 mediated signaling is increased to treat a disease wherein decreased apoptosis or decreased cytokine and adhesion molecule expression is exhibited. An agonist
5 can include soluble forms of DR3-V1 or DR3 and monoclonal antibodies directed against the DR3-V1 or DR3 polypeptide.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the, DR3-V1 or DR3 polypeptide an
10 effective amount of an antagonist capable of decreasing DR3-V1 or DR3 mediated signaling. Preferably, DR3-V1 or DR3 mediated signaling is decreased to treat a disease wherein increased apoptosis or NFkB expression is exhibited. An antagonist can include soluble forms of DR3-V1 or DR3 and monoclonal antibodies directed against the DR3-V1 or DR3 polypeptide.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family
15 ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such
20 an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound
25 is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in *Science* 246:181-296 (October 1989). For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

Another method involves screening for compounds which inhibit activation of the receptor polypeptide of the present invention antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA encoding the receptor such that the cell expresses the receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity of the receptors. If the compound binds to the receptor as determined by a reduction of labeled ligand which binds to the receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307(1992).

5 Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR3-V1 or DR3 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the DR3-V1 or DR3 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

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Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor β , neurotransmitters (such as glutamate, dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonist include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and β -amyloid peptide. (*Science* 267:1457-1458 (1995)). Further preferred agonist include polyclonal and monoclonal antibodies raised against the DR3-V1 or DR3 polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in Tartaglia, L.A., *et al.*, *Proc. Natl. Acad. Sci.*

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USA 88:9292-9296 (1991); and Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267 (7):4304-4307 (1992) See, also, PCT Application WO 94/09137.

Antagonist according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus *E1B*,
5 Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRF1*, *LMP-1*, African swine fever virus *LMW5-HL*, and Herpesvirus *yl 34.5*), calpain inhibitors, cysteine protease inhibitors, and tumor promoters (such as PMA, Phenobarbital, and α -Hexachlorocyclohexane).

10 Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, *J. Neurochem.* 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988).
15 Triple helix formation is discussed in, for instance Lee *et al.*, *Nucleic Acids Research* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the
20 mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo*
25 and blocks translation of the mRNA molecule into receptor polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the receptor.

Further antagonist according to the present invention include soluble
30 forms of DR3-V1 or DR3, i.e., DR3-V1 or DR3 fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such

soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize DR3-V1 or DR3 mediated signaling by competing with the cell surface DR3-V1 or DR3 for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting apoptosis induced by TNF-family ligands. These are preferably expressed as dimers or trimers, since these have been shown to be superior to monomeric forms of soluble receptor as antagonists, e.g., IgGFc-TNF receptor family fusions. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med.* 182:1395-1401 (1995)).

The experiments set forth in Examples 6 and 7 demonstrate that DR3 is a death domain-containing molecule capable of triggering both apoptosis and NF-kB activation, two pathways dominant in the regulation of the immune system. The experiments also demonstrate the internal signal transduction machinery of this novel cell death receptor. In addition, the experiments set forth below demonstrate that DR3-induced apoptosis was blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk. Importantly, apoptosis induced by DR3 was also blocked by dominant negative versions of FADD (FADD-DN) or FLICE (FLICE-DN/MACHa1C360S), which were previously shown to inhibit death signaling by Fas/APO-1 and TNFR-1. Thus, inhibitors of ICE-like proteases, FADD-DN and FLICE-DN/MACHa1C360S could also be used as antagonists for DR3 activity.

The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab')₂ fragments) which are capable of binding an antigen. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of methods using DR3-V1 or DR3 immunogens of the present

invention. As indicated, such DR3-V1 or DR3 immunogens include the full length DR3-V1 or DR3 polypeptide (which may or may not include the leader sequence) and DR3-V1 or DR3 polypeptide fragments such as the ligand binding domain, the transmembrane domain, the intracellular domain and the death domain.

Proteins and other compounds which bind the DR3-V1 or DR3 domains are also candidate agonist and antagonist according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature* 340:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (Gyuris, J. *et al.*, *Cell* 75:791-803 (1993); Zervos, A.S. *et al.*, *Cell* 72:223-232 (1993)). Preferably, the yeast two-hybrid system is used according to the present invention to capture compounds which bind to either the DR3-V1 or DR3 ligand binding domain or to the DR3-V1 or DR3 intracellular domain. Such compounds are good candidate agonist and antagonist of the present invention.

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor family and inducing the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, the DR3-V1 or DR3 ligand, TNF- α , lymphdotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40, CD27, CD30, 4-1BB, OX40 and nerve growth factor (NGF).

Representative therapeutic applications of the present invention are discussed in more detail below. The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of CD4⁺ T-lymphocytes. Recent reports estimate the daily loss of CD4⁺ T cells to be between 3.5×10^7 and 2×10^9 cells (Wei X., *et al.*, *Nature* 373:117-122 (1995)). One cause of CD4⁺ T cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis. Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (Ameisen, J.C., *AIDS* 8:1197-1213 (1994); Finkel, T.H., and Banda, N.K., *Curr.*

Opin. Immunol. 6:605-615(1995); Muro-Cacho, C.A. *et al.*, *J. Immunol.* 154:5555-5566 (1995)). Furthermore, apoptosis and CD4⁺ T-lymphocyte depletion is tightly correlated in different animal models of AIDS (Brunner, T., *et al.*, *Nature* 373:441-444 (1995); Gougeon, M.L., *et al.*, *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)) and, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (Gougeon, M.L. *et al.*, *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)). Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the TNF-family ligand FasL. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of U937 cells with HIV results in the *de novo* expression of FasL and that FasL mediates HIV-induced apoptosis (Badley, A.D. *et al.*, *J. Virol.* 70:199-206 (1996)). Further the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes (Badley, A.D *et al.*, *J. Virol.* 70:199-206 (1996)). Thus, by the invention, a method for treating HIV⁺ individuals is provided which involves administering an antagonist of the present invention to reduce selective killing of CD4 T-lymphocytes. Modes of administration and dosages are discussed in detail below.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. Agonist of the present invention

are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the DR3-V1 or DR3 polypeptide, and thereby are susceptible to compounds which enhance apoptosis. Thus, the present invention further provides a method for creating immune privileged tissues. Antagonist of the invention can further be used in the treatment of Inflammatory Bowel-Disease.

DR3, like TNFR1, also activates the NF-kB transcription factor, which is very closely associated with the stimulation of cytokine (e.g., IL-8) and adhesion molecule (e.g., ELAM) transcription. Hence, like TNF, the ligand (or agonist) for DR3 and DR3-V1 may in some circumstances be proinflammatory, and antagonists may be useful reagents for blocking this response. Thus, DR3 and DR3-V1 antagonists may be useful for treating inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

In addition, due to lymphoblast expression of DR3, soluble DR3, agonist or antagonist mABs may be used to treat this form of cancer. Further, soluble DR3 or neutralizing mABs may be used to treat various chronic and acute forms of inflammation such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

Modes of Administration

The agonist or antagonists described herein can be administered *in vitro*, *ex vivo*, or *in vivo* to cells which express the receptor of the present invention. By administration of an "effective amount" of an agonist or antagonist is intended an amount of the compound that is sufficient to enhance or inhibit a cellular response to a TNF-family ligand and include polypeptides. In particular, by administration of an "effective amount" of an agonist or antagonists is intended an amount effective to enhance or inhibit DR3-V1 or DR3 mediated apoptosis. Of course, where apoptosis is to be enhanced, an agonist according to the present invention can be co-administered with a TNF-family ligand. One of ordinary

skill will appreciate that effective amounts of an agonist or antagonist can be determined empirically and may be employed in pure form or in pharmaceutically acceptable salt, ester or prodrug form. The agonist or antagonist may be administered in compositions in combination with one or more pharmaceutically acceptable excipients.

It will be understood that, when administered to a human patient, the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgement. The specific therapeutically effective dose level for any particular patient will depend upon factors well known in the medical arts.

As a general proposition, the total pharmaceutically effective amount of DDCR polypeptide administered parenterally per dose will be in the range of about 1 $\mu\text{g/kg/day}$ to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day , and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the DDCR agonists or antagonists is typically administered at a dose rate of about 1 $\mu\text{g/kg/hour}$ to about 50 $\mu\text{g/kg/hour}$, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed.

Dosaging may also be arranged in a patient specific manner to provide a predetermined concentration of an agonist or antagonist in the blood, as determined by the RIA technique. Thus patient dosaging may be adjusted to achieve regular on-going trough blood levels, as measured by RIA, on the order of from 50 to 1000 ng/ml , preferably 150 to 500 ng/ml .

Pharmaceutical compositions are provided comprising an agonist or antagonist and a pharmaceutically acceptable carrier or excipient, which may be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. Importantly, by co-administering an agonist and a TNF-family ligand clinical side effects can be reduced by using

lower doses of both the ligand and the agonist. It will be understood that the agonist can be "co-administered" either before, after, or simultaneously with the TNF-family ligand, depending on the exigencies of a particular therapeutic application. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

In addition to soluble DR3-V1 or DR3 polypeptides, DR3-V1 or DR3 polypeptide containing the transmembrane region can also be used when appropriately solubilized by including detergents, such as CHAPS or NP-40, with buffer.

Example 1

Expression and Purification in E. coli

The DNA sequence encoding the mature DR3-V1 protein in the deposited cDNA clone (ATCC No. 97456) is amplified using PCR oligonucleotide primers specific to the amino terminal sequences of the DR3-V1 protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The following primers are used for expression of DR3 extracellular domain in *E. coli* 5' primer 5'-GCGCCATGGGGGCCCCGGCGGCAG-3' (SEQ ID NO:7) contains an NcoI site and 15 nucleotide starting from 290 nucleotide to 304 FIG. 1. 3' primer 5'-GCGAAGCTTCTAGGACCCAGAACATCTGCC-3'

complimentary to nucleotide from 822 to 840 in FIG. 1. Vector is pQE60. The protein is not tagged.

The restriction sites are convenient to restriction enzyme sites in the bacterial expression vector pQE60, which are used for bacterial expression in these examples. (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS").

The amplified DR3-V1 DNA and the vector pQE60 both are digested with Nco I and HindIII and the digested DNAs are then ligated together. Insertion of the DDCR protein DNA into the restricted pQE60 vector places the DR3-V1 protein coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-frame with an initiating AUG appropriately positioned for translation of DR3-V1 protein.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing DR3-V1 protein, is available commercially from Qiagen.

Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells are grown to an optical density at 600nm ("OD₆₀₀") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside

("IPTG") is then added to a final concentration of 1 mM to induce transcription from *lac* repressor sensitive promoters, by inactivating the *lacI* repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation and disrupted, by standard methods. Inclusion bodies are purified from the disrupted cells using routine collection techniques, and protein is solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein is passed over a PD-10 column in 2X phosphate-buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein is purified by a further step of chromatography to remove endotoxin. Then, it is sterile filtered. The sterile filtered protein preparation is stored in 2X PBS at a concentration of 95 μ /ml.

Example 2

Expression in Mammalian Cells

Most of the vectors used for the transient expression of a given gene sequence in mammalian cells carry the SV40 origin of replication. This allows the replication of the vector to high copy numbers in cells (e.g. COS cells) which express the T antigen required for the initiation of viral DNA synthesis. Any other mammalian cell line can also be utilized for this purpose.

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g. RSV, HTLV, HIV and the early promoter of the cytomegalovirus (CMV). However, also cellular signals can be used (e.g. human actin, promoter). Suitable expression vectors for use in practicing the present invention include, for

pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC67109). Mammalian host cells that could be used include, human HeLa, 283, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1 African green monkey cells, quail QC1-3 cells, mouse L cells and Chinese hamster ovary cells such as

Alternatively, a gene of interest can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) is a useful marker to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Using this marker, the mammalian cells are grown in increasing amounts of methotrexate for selection and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) cells are often used for the production of proteins.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology* 438:44701 (March 1985)), plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g. with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Example 2A

Expression of extracellular soluble domain of DR3-V1 and DR3 in COS cells

The expression plasmid, pDR3-V1 HA, is made by cloning a cDNA encoding DR3-V1 (ATCC No. 97456) into the expression vector pcDNA1/Amp

is made by cloning a cDNA encoding DR3 (ATCC No. _____) into the expression vector pcDNAI/Amp

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cell; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron, and a polyadenylation signal arranged so that a cDNA conveniently can be placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker.

A DNA fragment encoding the entire DR3-V1 or Dr3 precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson *et al.*, *Cell* 37:767 (1984). The fusion of the HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is as follows:

The DR3-V1 or DR3 cDNA of the deposit clone is amplified using primers that contained convenient restriction sites, much as described above regarding the construction of expression vectors for expression of DR3-V1 or DR3 in *E. coli* and *S. fugiperda*.

To facilitate detection, purification and characterization of the expressed DR3-V1 or DR3, one of the primers contains a hemagglutinin tag ("HA tag") as described above.

Suitable primers for DR3-V1 include the following, which are used in this example, the 5' primer, 5' CGCGGATCCGCCATCATGGAGGAGACGCAGCAG 3' (SEQ ID NO:9) contains the underlined BamHI site, an ATG start codon and 5 codons thereafter.

Suitable primers for DR3 include the following, which are used in this example, the 5' primer, 5'

CGCGGATCCGCCATCATGGAGCAGCGGCCGCGG 3' (SEQ ID NO:10)
contains the underlined BamHI site, an ATG start codon and 5 codons thereafter.

The 3' primer for both DR3 and DR3-V1, containing the underlined XbaI
site, stop codon, hemagglutinin tag and last 14 nucleotide of 3' coding sequence
(at the 3' end) has the following sequence:

5'GCGTCTAGATCAAAGCGTAGTCTGGGACGTCGTATGGGTACGGGC
CGCGCTGCA 3' (SEQ ID NO:11).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are
digested with BamHI and XbaI and then ligated. The ligation mixture is
transformed into *E. coli* strain SURE (available from Stratagene Cloning
Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed
culture is plated on ampicillin media plates which then are incubated to allow
growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant
colonies and examined by restriction analysis and gel sizing for the presence of
the DR3-V1 or DR3-encoding fragment.

For expression of recombinant DR3-V1 or DR3, COS cells are transfected
with an expression vector, as described above, using DEAE-DEXTRAN, as
described, for instance, in Sambrook *et al.*, *Molecular Cloning: a Laboratory
Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, NY (1989).

Cells are incubated under conditions for expression of DR3-V1 or DR3
by the vector.

Expression of the DR3-V1 HA fusion protein or the DR3 HA fusion
protein is detected by radiolabelling and immunoprecipitation, using methods
described in, for example Harlow *et al.*, *Antibodies: a Laboratory Manual*, 2nd
Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988). To
this end, two days after transfection, the cells are labeled by incubation in media
containing ³⁵S-cysteine for 8 hours. The cells and the media are collected, and the
cells are washed and then lysed with detergent-containing RIPA buffer: 150 mM
NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as
described by Wilson *et al.* cited above. Proteins are precipitated from the cell

The precipitated proteins then are analyzed by SDS-PAGE gels and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 2B

5 *Expression and purification of human DR3-V1 and DR3 using the CHO Expression System*

10 The vector pC1 is used for the expression of DR3-V1 or DR3 (ATCC No. 97456 or ATCC No. _____, respectively) protein. Plasmid pC1 is a derivative of the plasmid pSV2-dhfr [ATCC Accession No. 37146]. Both plasmids contain the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F.W., Kellems, R.M., Bertino, J.R., and Schimke, R.T., 1978, J. Biol. Chem. 253:1357-1370, Hamlin, J.L. and Ma, C. 1990, Biochem. et Biophys. Acta, 1097:107-143, Page, M.J. and Sydenham, M.A. 1991, Biotechnology Vol. 9:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene it is usually co-amplified and over-expressed. It is state of the art to develop cell lines carrying more than 1,000 copies of the genes. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome(s).

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Plasmid pC1 contains for the expression of the gene of interest a strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, *et al.*, Molecular and Cellular Biology, March 1985:438-4470) plus a fragment

cytomegalovirus (CMV) (Boshart *et al.*, *Cell* 41:521-530, 1985). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI followed by the 3' intron and the polyadenylation site of the rat preproinsulin gene. Other high efficient promoters can also be used for the expression, e.g., the human β -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pCI is digested with the restriction enzyme BamHI and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding DR3-V1 or DR3 in the deposited cDNA clones are amplified using PCR oligonucleotide primers specific to the amino acid carboxyl terminal sequence of the DR3-V1 or DR3 protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The 5' oligonucleotide primer for DR3-V1 has the sequence 5' CGCGGATCCGCCATCATGGAGGAGACGCAGCAG 3' (SEQ ID NO:12) containing the underlined BamHI restriction site, which encodes a start AUG, followed by the Kozak sequence ad 18 nucleotides of the DR3-V1 coding sequence set out in FIG. 1 beginning with the 1st base of the ATG codon.

The 5' oligonucleotide primer for DR3 has the sequence 5' CGCGGATCCGCCATCATGGAGCAGCGGCCGCGG 3' (SEQ ID NO:13) containing the underlined BamHI restriction site, which encodes a start AUG, followed by the Kozak sequence ad 18 nucleotides of the DR3 coding sequence set out in FIG. 2 beginning with the 1st base of the ATG codon.

The 3' primer for both DR3 and DR3-V1 has the sequence
5' CGCGGATCCTCACGGGCCGCGCTGCA 3' (SEQ ID NO:14) containing the
underlined BamHI restriction site followed by 17 nucleotides complementary to
the last 14 nucleotides of the DR3-V1 or DR3 coding sequence set out in FIG. 1
or FIG. 2, respectively, plus the stop codon.

The restriction sites are convenient to restriction enzyme sites in the
CHO expression vectors pC1.

The amplified DR3 or DR3-V1 DNA and the vector pC1 both are digested
with BamHI and the digested DNAs then ligated together. Insertion of the DR3-
V1 or DR3 DNA into the BamHI restricted vector placed the DR3-V1 or DR3
coding region downstream of and operably linked to the vector's promoter. The
sequence of the inserted gene is confirmed by DNA sequencing.

Transfection of CHO-DHFR-cells

Chinese hamster ovary cells lacking an active DHFR enzyme are used for
transfection. 5 µg of the expression plasmid C1 are cotransfected with 0.5 µg of
the plasmid pSVneo using the lipofecting method (Felgner *et al.*, *supra*). The
plasmid pSV2-neo contains a dominant selectable marker, the gene neo from Tn5
encoding an enzyme that confers resistance to a group of antibiotics including
G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml
G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning
plates (Greiner, Germany) and cultivated from 10-14 days. After this period,
single clones are trypsinized and then seeded in 6-well petri dishes using different
concentrations of methotrexate (25 nM, 50 nM, 100 nM, 200 nM, 400 nM).
Clones growing at the highest concentrations of methotrexate are then transferred
to new 6-well plates containing even higher concentrations of methotrexate (500
nM, 1 µM, 2 µM, 5 µM). The same procedure is repeated until clones grow at
a concentration of 100 µM.

The expression of the desired gene product is analyzed by Western blot
analysis and SDS-PAGE.

Example 3

Cloning and expression of the soluble extracellular domain of DR3-V1 and DR3 in a baculovirus expression system

The cDNA sequence encoding the soluble extracellular domain of DR3-V1 or DR3 protein in the deposited clone (ATCC No. 97456 or ATCC No. _____, respectively) is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer for DR3-V1 has the sequence 5' CGCGGATCC GCCATCATGGAGGAGACGCAGCAG 3' (SEQ ID NO:15) containing the underlined BamHI restriction enzyme site followed by Kozak sequence and a number of bases of the sequence of DR3-V1 of FIG. 1. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DR3-V1 provides an efficient signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.* 196:947-950 (1987) is appropriately located in the vector portion of the construct.

The 5' primer for DR3 has the sequence 5' CGCGGATCC GCCATCATGGAGCAGCGGCCGCGG 3' (SEQ ID NO:16) containing the underlined BamHI restriction enzyme site followed by Kozak sequence and a number of bases of the sequence of DR3 of FIG. 2. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DR3 provides an efficient signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.* 196:947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer for both DR3 and DR3-V1 has the sequence 5' GCGAGATCTAGTCTGGACCC AGAACATCTGCCTCC 3' (SEQ ID NO:17) containing the underlined XbaI restriction followed by nucleotides complementary to the DR3-V1 or DR3 nucleotide sequence set out in FIG. 1 or FIG.2, respectively, followed by the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("GeneClean, " BIO 101 Inc., La Jolla, Ca.) The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel. This fragment is designated herein F2.

5 The vector pA2 is used to express the DR3-V1 or DR3 protein in the baculovirus expression system, using standard methods, such as those described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedron promoter of the
10 Autograph californica nuclear polyhedrosis virus (ACMNPV) followed by convenient restriction sites. For an easy selection of recombinant virus the beta-galactosidase gene from *E. coli* is inserted in the same orientation as the polyhedron promoter and is followed by the polyadenylation signal of the polyhedron gene. The polyhedron sequences are flanked at both sides by viral
15 sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of pA2, such as pAc373, pVL941 and pAcIM1 provided, as those of skill readily will appreciate, that construction provides appropriately located signals for transcription,
20 translation, trafficking and the like, such as an in-frame AUG and a signal peptide, as required. Such vectors are described in Luckow *et al.*, *Virology* 170:31-39, among others.

The plasmid is digested with the restriction enzymes Bam HI and XbaI and then is dephosphorylated using calf intestinal phosphatase, using routine
25 procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V2".

Fragment F2 and the dephosphorylated plasmid V2 are ligated together with T4 DNA ligase. *E. coli* HB101 cells are transformed with ligation mix and
30 spread on culture plates. Bacteria are identified that contain the plasmid with the human DDCR gene by digesting DNA from individual colonies using BamHI and

XbaI and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac DR3-V1 or pBac DR3.

5 5 µg of the plasmid pBac DR3-V1 or pBac DR3 is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987). 1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac DR3-V1 are mixed in a sterile well of a microliter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

15 After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

20 Four days after serial dilution, the virus is added to the cells. After appropriate incubation, blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four

days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. A clone containing properly inserted DR3-V1 or DR3 is identified by DNA analysis including restriction mapping and sequencing. This is designated herein as V- DR3-V1 or V-DR3.

5 Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-DR3-V1 at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc.,
10 Gaithersburg). 42 hours later, 5 gCi of ³⁵S-methionine and 5 μCi ³⁵S cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation, lysed and the labeled proteins are visualized by SDS-PAGE and autoradiography.

Example 4

15 A. *Tissue distribution of DR3-V1 gene expression*

Northern blot analysis is carried out to examine DR3-V1 gene (ATCC No. 97456) expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the DR3-V1 protein (SEQ ID NO:1) is labeled with ³²P
20 using the *rediprime*TM DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100TM column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for DR3-V1 mRNA.

25 Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with labeled probe using *ExpressHyb*TM hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following

hybridization and washing, the blots are mounted and exposed to film at -70°C overnight, and films developed according to standard procedures. Expression of DR3-V1 was detected in tissues enriched in lymphocytes including peripheral blood leukocytes (PBLs), thymus, spleen, colon, and small intestine. DR3-V1 expression appears to be restricted to lymphocyte compartments, it can be envisaged that DR3-V1 plays a role in lymphocyte homeostasis.

B. Tissue distribution of DR3 gene expression

Northern blot analysis is carried out to examine DR3 gene (ATCC No. _____) expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the DR3 protein (SEQ ID NO:1) is labeled with ³²P using the rediprime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for DR3 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70°C overnight, and films developed according to standard procedures.

Expression of DR3 was detected in tissues enriched in lymphocytes including peripheral blood leukocytes (PBLs), thymus, spleen, colon, and small intestine. By contrast, TNFR-1 is ubiquitously expressed and Fas/APO-1 is expressed in lymphocytes, liver, heart, lung, kidney, and ovary (Watanabae-Fukunaga, *et al.*, *J. Immunol* 148:1274-9 (1992)).

DR3 expression appears to be restricted to lymphocyte compartments, it can be envisaged that DR3 plays a role in lymphocyte homeostasis.

C. Northern Blot analysis of DR3 in various cell lines

Methods

Cells

Unless stated otherwise, cell lines were obtained from the American Type Culture Collection (Rockville, MD). The myeloid (Koeffler *et al.* (1980); Koeffler (1983); Harris and Ralph (1985); and Tucker *et al.* (1987) and B-cell lines (Jonak *et al.* (1922)) studied represent cell types at different stages of the differentiation pathway. KG1a and PLB 985 cells (Tucker *et al.* (1987)) were obtained from H.P. Koeffler (UCLA School of Medicine). BJA-B was from Z. Jonak (SmithKline Beecham). TF274, a stromal cell line exhibiting osteoblastic features, was generated from the bone marrow of a healthy male donor (Z. Jonak and K.B. Tan, unpublished). Primary carotid artery endothelial cells were purchased from Clonetics Corp. (San Diego, CA) and monocytes were prepared by differential centrifugation of peripheral blood mononuclear cells and adhesion to tissue culture dish. CD19+, CD4+ and CD8+ cells (>90% pure) were isolated with cell type specific immunomagnetic beads (Dynal, Lake Success, NY).

RNA Analysis

Total RNA of adult tissues were purchased from Clontech (Palo Alto, CA). Total RNA was extracted from cell lines (in exponential growth phase) and primary cells with TriReagent (Molecular Research Center, Inc., Cincinnati, OH). 5 to 7.5 µg of total RNA was fractionated in a 1% agarose gel containing formaldehyde cast in a Wide Mini-Sub Cell gel tray (Bio-Rad, Hercules, CA) as described (Sambrook, *et al.*) with slight modifications. The formaldehyde concentration was reduced to 0.5M and the RNA was stained prior to electrophoresis with 100 µg/ml of etidium bromide that was added to the loading buffer. After electrophoresis with continuous buffer recirculation (60 volts/90 min), the gel was photographed and the RNA was transferred quantitatively to Zeta-probe nylon membrane (Biorad, Hercules, CA) by vacuum-blotting with 25

mM NaOH for 90 min. After neutralization for 5-10 min, with 1M Tris-HCl, pH 7.5 containing 3M NaCl, the blots were prehybridized with 50% formamide, 8% dextran sulfate, 6xSSPE, 0.1% SDS and 100 µg/ml of sheared and denatured salmon sperm DNA for at least 30 min at 42°C. cDNA inserts labeled with ³²P-dCTP by random priming (Stratagene, La Jolla, CA), were denatured with 0.25M NaOH (10 min at 37°C) and added to the prehybridization solution. After 24-65 hr at 42°C, the blots were washed under high stringency conditions (Sambrook, et al.) and exposed to X-ray films.

Results

Expression of DR3 was assessed by Northern blot in the following cell lines: TF274 (bone marrow stromal); MG63, TE85 (osteosarcoma); K562 (erythroid); KG1a, KG1, PLB985, HL60, U937, TNHP-1 (myeloid); REH, BJAB, Raji, IM-9 (B cell); Sup-T1, Jurkat, H9, Molt-3 (T cell); RL95-2 (endometrial carcinoma); MCF-7 (breast cancer); BE, HT29 (colon cancer); IMR32 (neuroblastoma) and could only be detected in KG1a cells. DR3 expression was detected in several lymphoblast cell lines. In the purified human hematopoietic cell populations, DR3 was weakly expressed in CD19+ cells, and more highly expressed in monocytes. However the highest levels were observed in T cells (CD4+ or CD8+) upon stimulation with PMA and PHA, indicating that DR3 probably plays a role in the regulation of T cell activation.

Example 5

Intracellular Signaling Molecules used by DR3 Protein

In vitro and *in vivo* binding studies were undertaken to investigate DR3 signaling pathways. Since DR3 contains a death domain, the inventors postulated that DR3, like TNFR-1 and Fas/APO-1, may transduce signals by recruiting death domain-containing adapter molecules (DAMs) such as FADD, TRADD, and RIP.

Experimental Design

In vitro binding experiments were performed as described previously (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)). Briefly, the cytoplasmic domains of DR3 (amino acid residues 215-393 (Figure 2)) and the death domain mutant Δ DR3 (amino acid residues 215-321 (Figure 2)) were amplified by PCR using appropriate templates and primers into pGSTag. pGSTag and pGSTag-TNFR-1 were described previously (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)). GST and GST fusion proteins were prepared from E.coli strain BL21(DE3)pLysS using standard published procedures and the recombinant proteins immobilized onto glutathione-agarose beads. ³⁵S-Labeled FADD, RIP and TRADD were prepared by *in vitro* transcription-translation using the TNT or T7 or SP6-coupled reticulocyte lysate system from Promega according to manufacturer's instructions, using pcDNA3 AU1-FADD (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)), pRK myc-TRADD (H. Hsu, *et al.*, *Cell* 81, 495-504 (1995)), or pRK myc-RIP (H. Hsu, *et al.*, *Immunity* 4, 387-396 (1996)) as template. Following translation, equal amounts of total ³⁵S-labeled reticulocyte lysate were diluted into 150 μ l GST binding buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 1% NP-40) and incubated for 2 hrs. at 4°C with the various GST fusion proteins complexed to beads, following the beads were pelleted by plus centrifugation, washed three times in GST buffer, boiled in SDS-sample buffer and resolved on a 12.5% SDS-PAGE. Bound proteins were visualized following autoradiography at -80°C. *In vitro* translated ³⁵S-labeled RIP, TRADD and FADD were incubated with glutathione beads containing GST alone or GST fusions of the cytoplasmic domain of Fas, TNFR-1, DR3 (215-393), or Δ DR3 (215-321). After the beads were washed, retained proteins were analyzed by SDS-PAGE and autoradiography. The gel was Coomassie stained to monitor equivalency of loading.

To demonstrate the association of DR3 and TRADD in vivo, constructs encoding Flag-TNFR-1 and Flag-ΔTNFR-1 were used. The Flag-TNFR-1 and Flag-ΔTNFR-1 constructs were described elsewhere (A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). The constructs encoding Flag-TNFR-1 and Flag-ΔTNFR-1 were described elsewhere (A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). To facilitate epitope tagging, DR3 and ΔDR3 (1-321) were cloned into the IBI Kodak FLAG plasmid (pCMV1FLAG) utilizing the signal peptide provided by the vector. 293 cells (2 x 10⁶/100mm plate) were grown in DMEM media containing 10% heat-inactivated fetal bovine serum containing penicillin G, streptomycin, glutamine, and non-essential amino acids. Cells were transfected using calcium phosphate precipitation with the constructs encoding the indicated proteins in combination with pcDNA3-CrmA (M. Tewari, *et al.*, *J Biol Chem* 270, 3255-60 (1995)) to prevent cell death and thus maintain protein expression. Cells were lysed in 1 ml lysis buffer (50mM Hepes, 150mM NaCl, 1mM EDTA, 1% NP-40, and a protease inhibitor cocktail). Lysates were immunoprecipitated with a control monoclonal antibody or anti-Flag antibody for at least 4 hrs, at 4°C as previously described (A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). The beads were washed with lysis buffer 3X, but in the case of TRADD binding, the NaCl concentration was adjusted to 1M. The precipitates were fractioned on 12.5% SDS-PAGE and transferred to nitrocellulose. Subsequent Western blotting was performed as described elsewhere (H. Hsu *et al.*, *Cell* 84, 299-308 (1996); Chinnaiyan, A.M. *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). After 24-32 hrs, extracts were prepared and immunoprecipitated with a control monoclonal antibody or anti-Flag monoclonal antibody (IBI Kodak). Western analysis indicated that myc-TRADD and death receptor expression levels were similar in all samples. Coprecipitating myc-TRADD was detected by immunoblotting using an anti-myc HRP conjugated antibody (Boehringer Mannheim).

Results

As an initial screen, *in vitro* translated radiolabeled DAMs were precipitated with various glutathione S-transferase (GST) fusion proteins immobilized on glutathione-Sepharose beads. As predicted from previous studies (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995); H. Hsu, *et al.*, *Cell* 81, 495-504 (1995)), FADD associated with the GST-Fas cytoplasmic domain while TRADD associated with the GST-TNFR-1 cytoplasmic domain. In addition, there was a direct, albeit weak, interaction between RIP and GST-TNFR-1. Interestingly, GST-DDCR associated specifically with TRADD, but not FADD or RIP. Furthermore, a truncated death domain mutant of DR3 (GST-DDR3) failed to interact with TRADD. To demonstrate the association of DR3 and TRADD *in vivo*, 293 cells were transiently transfected with plasmids that direct the synthesis of myc-epitope tagged TRADD (myc-TRADD) and Flag-epitope tagged DR3 (Flag-DR3), Flag-TNFR-1 or mutants. Consistent with the *in vitro* binding study, TRADD specifically coprecipitated with DR3 and TNFR-1, but not with the death domain mutants, DDR3 and DTNFR-1. Thus, it appears that DR3, like TNFR-1, may activate downstream signaling cascades by virtue of its ability to recruit the adapter molecule TRADD.

Overexpression of TRADD induces apoptosis and NF- κ B activation-two of the most important activities signaled by TNFR-1 (H. Hsu, *et al.*, *Cell* 81, 495-504 (1995)). Upon oligomerization of TNFR-1 by trimeric TNF, TRADD is recruited to the receptor signaling complex (H. Hsu, *et al.*, *Cell* 84, 299-308 (1996)). TRADD can then recruit the following signal transducing molecules: 1) TRAF2, a TNFR-2- and CD40 - associated molecule (M. Rothe, *et al.*, *Cell* 78, 681-92 (1994); M. Rothe, *et al.*, *Science* 269, 1424-1427 (1995)), that mediates NF- κ B activation, 2) RIP, originally identified as a Fas/APO-1-interacting protein by two-hybrid analysis (B.Z. Stanger, *et al.*, *Cell* 81, 513-23 (1995)), that mediates NF- κ B activation and apoptosis (H. Hsu, *et al.*, *Immunity* 4, 387-396 (1996)), and 3) FADD, a Fas/APO-1- associated molecule, that mediates

apoptosis (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J. Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)). Thus, the inventors demonstrate that RIP, TRAF2 and FADD could be co-immunoprecipitated with DR3. In 293 cells expressing DR3 and RIP, only a weak association could be detected between the two molecules. However, in the presence of TRADD, RIP association with DR3 was significantly enhanced. Likewise, very little TRAF2 directly co-precipitated with DR3 in 293 cells. However, when DR3 and TRAF2 were expressed in the presence of TRADD and RIP (both of which can bind TRAF2), an enhanced binding of TRAF2 to DR3 could be detected. A similar association between FADD and DR3 was also observed. In the presence of TRADD, FADD efficiently coprecipitated with DR3.

Previous studies demonstrated that FADD could recruit the ICE/CED-3-like protease FLICE to the Fas/APO-1 death inducing signaling complex (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M.P. Boldin, *et al.*, *Cell* 85, 803-815 (1996)). To demonstrate that FLICE can associate with TNFR-1 and DR3, coprecipitation experiments in 293 cells were carried out. Interestingly, FLICE was found complexed to TNFR-1 and DR3. Co-transfection of TRADD and/or FADD failed to enhance the FLICE-TNFR-1/DR3 interaction, suggesting that endogenous amounts of these adapter molecules were sufficient to maintain this association.

Example 6

DR3 Induced Apoptosis and NF-kB Activation

Overexpression of Fas/APO-1 and TNFR-1 in mammalian cells mimics receptor activation (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin, *et al.*, *Cell* 85, 803-815 (1996)). Thus, this system was utilized to study the functional role of DDCR. Ectopic expression of DR3 in MCF7 breast carcinoma cells and 293 human embryonic kidney cells induced rapid apoptosis.

Experimental Design

Cell death assays were performed essentially as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995);
5 A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). Briefly, MCF-7 human breast carcinoma clonal cell lines stably transfected with either vector alone, a CrmA expression construct (M. Tewari, *et al.*, *J Biol Chem* 270, 3255-60 (1995)), or FADD-DN expression construct (A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)) were transiently transfected with pCMV- β -galactosidase in the presence of a ten-fold excess of pcDNA3 expression constructs encoding the indicated proteins using lipofectamine (GIBCO-BRL).
10 293 cells were likewise transfected using the CaPO₄ method. The ICE family inhibitor z-VAD-fmk (Enzyme Systems Products, Dublin, CA) was added to the cells at a concentration of 10 μ M, 5 hrs after transfection. 32 hours following
15 transfection, cells were fixed and stained with X-Gal as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)). The data (mean \pm SD) shown are the percentage of round blue cells among the total number of blue cells counted. Data were obtained from at least three
20 independent experiments.

NF- κ B luciferase assays were performed as described elsewhere (H. Hsu, *et al.*, *Immunity* 4, 387-396 (1996); M.D. Adams, *et al.*, *Nature* 377, 3-174 (1995); G.S. Feng, *et al.*, *J Biol Chem* 271, 12129-32 (1996); M. Rothe, *et al.*, *Cell* 78, 681-92 (1994); M. Rothe, *et al.*, *Science* 269, 1424-1427 (1995); (A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). Briefly, 293 cells were
25 co-transfected by calcium phosphate precipitation with pCMV- β -galactosidase, E-selectin-luciferase reporter gene (M. Rothe, *et al.*, *Cell* 78, 681-92 (1994); M. Rothe, *et al.*, *Science* 269, 1424-1427 (1995)), the indicated death receptors, and the indicated dominant negative inhibitors. In addition, DR3 or DDR3 was
30 cotransfected with the pLantern expression construct (GIBCO-BRL) which encodes green fluorescent protein (photographic inset). Cells were visualized by

fluorescence microscopy using a FITC range barrier filter cube. Nuclei of transfected cells were visualized by DAPI staining and the image overlaid. (Cell death assays were performed essentially as previously described (Chinnaiyan, *et al.*, *Cell* 81:505-12 (1995); Boldin, *et al.*, *J. Biol. Chem.* 270:7795-8 (1995); Kischkel, *et al.*, *EMBO* 14:5579-5588 (1995)); (Chinnaiyan, *et al.*, *J. Biol. Chem.* 271:4961-4965 (1996)). The dominant negative inhibitors were used at a 4-fold higher quantity than the death receptors. Total DNA was kept constant.

To show that DR3 induces NF- κ B activation which is inhibitable by RIP-DN (Stanger, *et al.*, *Cell* 81:513-23 (1995)) and TRAF2-DN (Hsu, *et al.* *Cell* 81:495-504 (1995)); (Rothe, *et al.*, *Cell* 78:681-92 (1994); Rothe, *et al.* *Science* 269:1424-1427 (1995)), 293 cells were co-transfected with the indicated molecules and an NF- κ B luciferase reporter plasmid (Rothe, *et al.*, *Cell* 78:681-92 (1994); Rothe, *et al.* *Science* 269:1424-1427 (1995)) and luciferase activities subsequently determined. NF- κ B luciferase assays were performed as described elsewhere ((Hsu, *et al.*, *Immunity* 4:387-396 (1996)); (Adams, *et al.*, *Nature* 377:3-174 (1995); Feng, *et al.*, *J. Biol. Chem.* 271:12129-32 (1996)); (Rothe, *et al.*, *Cell* 78:681-92 (1994); Rothe, *et al.* *Science* 269:1424-1427 (1995)); Chinnaiyan, *et al.*, *J. Biol. Chem.* 271:4961-4965 (1996)). Briefly, 293 cells were co-transfected by calcium phosphate precipitation with pCMB- β -galactosidase, E-selectin-luciferase reporter gene (Rothe, *et al.*, *Cell* 78:681-92 (1994); Rothe, *et al.* *Science* 269:1424-1427 (1995)), the indicated death receptors, and the indicated dominant negative inhibitors. The dominant negative inhibitors were used at a 4-fold higher quantity than the death receptors. Total DNA was kept constant. Representative experiment performed in duplicate three independent times (mean \pm SD).

Results

The cells displayed morphological alterations typical of cells undergoing apoptosis, becoming rounded, condensed and detaching from the dish. In MCF7 cells, plasmids encoding full-length DR3 or DDR3 were co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells

transfected with DR3, but not DDR3, exhibited apoptotic morphology as assessed by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin, *et al.*, *Cell* 85, 803-815 (1996); M. Tewari, *et al.*, *J Biol Chem* 270, 3255-60 (1995)), DR3-induced apoptosis was blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk. Importantly, apoptosis induced by DR3 was also blocked by dominant negative versions of FADD (FADD-DN) or FLICE (FLICE-DN/MACHa1C360S), which were previously shown to inhibit death signaling by Fas/APO-1 and TNFR-1 (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin, *et al.*, *Cell* 85, 803-815 (1996); H. Hsu, *et al.*, *Cell* 84, 299-398 (1996); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). Thus, FADD and the ICE-like protease FLICE are likely necessary components of DR3-induced apoptosis.

As DR3 activation recruits three molecules implicated in TNF-induced NF-kB activation, we examined whether DR3 could activate NF-kB. Transfection of a control vector or expression of Fas/APO-1 failed to induce NF-kB activation. By contrast, NF-kB was activated by ectopic expression of DR3 or TNFR-1, but not by the inactive signaling mutants DDR3 or DTNFR-1. Importantly, DR3-induced NF-kB activation was blocked by dominant negative derivatives of RIP (RIP-DN) and TRAF2 (TRAF2-DN), which were previously shown to abrogate TNF-induced NF-kB activation (H. Hsu, *et al.*, *Cell* 84, 299-398 (1996); H. Hsu, *et al.*, *Immunity* 4, 387-396 (1996)). As expected, FADD-DN did not interfere with DR3-mediated NF-kB activation (H. Hsu, *et al.*, *Cell* 84, 299-398 (1996); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)).

Thus, the experiments set forth in Examples 6 and 7 demonstrate that DR3 is a death domain-containing molecule capable of triggering both apoptosis and NF-kB activation, two pathways dominant in the regulation of the immune system. The experiments also demonstrate the internal signal transduction machinery of this novel cell death receptor. The DR3 signaling complex assembles in a hierarchical manner with the recruitment of the multivalent

1) NF-kB activation mediated by TRAF2 and RIP and 2) cell death mediated by FADD, FLICE, and RIP.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

5 Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosures of all patents, patent applications, and publications referred to herein are hereby incorporated by reference.

SEQUENCE LISTING

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1783 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
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- (A) NAME/KEY: CDS
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATGGGTGGG GGTGGGGGCG CTGCTGGATT CCTGCTCTGG TGGAGGGGAA ACTTGTGAGG	60
GGCTGGTAAG CGCCCCCTCC GAAGCCTGGT GTGTGCGCGG GGGGAAGGAA GTTAGTTTCC	120
TCTCCACCCA TGGGCACCCC TTCTGCCCCG GGCCTGGGAA GTGGGCTGCT CTGTGGGCAA	180
ATGCTGGGGC CTCTGAA ATG GAG GAG ACG CAG CAG GGA GAG GCC CCA CGT	230
Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg	
1 5 10	
GGG CAG CTG CGC GGA GAG TCA GCA GCA CCT GTC CCC CAG GCG CTC CTC	278
Gly Gln Leu Arg Gly Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu	
15 20 25	
CTG GTG CTG CTG GGG GCC CGG GCC CAG GGC GGC ACT CGT AGC CCC AGG	326
Leu Val Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg	
30 35 40	
TGT GAC TGT GCC GGT GAC TTC CAC AAG AAG ATT GGT CTG TTT TGT TGC	374
Cys Asp Cys Ala Gly Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys	
45 50 55	
AGA GGC TGC CCA GCG GGG CAC TAC CTG AAG GCC CCT TGC ACG GAG CCC	422
Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro	
60 65 70 75	
TGC GGC AAC TCC ACC TGC CTT GTG TGT CCC CAA GAC ACC TTC TTG GCC	470
Cys Gly Asn Ser Thr Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala	
80 85 90	
TGG GAG AAC CAC CAT AAT TCT GAA TGT GCC CGC TGC CAG GCC TGT GAT	518
Trp Glu Asn His His Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp	
95 100 105	
GAG CAG GCC TCC CAG GTG GCG CTG GAG AAC TGT TCA GCA GTG GCC GAC	566
Glu Gln Ala Ser Gln Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp	
110 115 120	
ACC CGC TGT GGC TGT AAG CCA GGC TGG TTT GTG GAG TGC CAG GTC AGC	614
Thr Arg Cys Gly Cys Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser	
125 130 135	
CAA TGT GTC AGC AGT TCA CCC TTC TAC TGC CAA CCA TGC CTA GAC TGC	662
Gln Cys Val Ser Ser Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys	
140 145 150 155	
GGG GCC CTG CAC CGC CAC ACA CGG CTA CTC TGT TCC CGC AGA GAT ACT	710
Gly Ala Leu His Arg His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr	

160	165	170	
GAC TGT GGG ACC TGC CTG CCT GGC TTC TAT GAA CAT GGC GAT GGC TGC Asp Cys Gly Thr Cys Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys 175 180 185			758
GTG TCC TGC CCC ACG AGC ACC CTG GGG AGC TGT CCA GAG CGC TGT GCC Val Ser Cys Pro Thr Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala 190 195 200			806
GCT GTC TGT GGC TGG AGG CAG ATG TTC TGG GTC CAG GTG CTC CTG GCT Ala Val Cys Gly Trp Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala 205 210 215			854
GGC CTT GTG GTC CCC CTC CTG CTT GGG GCC ACC CTG ACC TAC ACA TAC Gly Leu Val Val Pro Leu Leu Leu Gly Ala Thr Leu Thr Tyr Thr Tyr 220 225 230 235			902
CGC CAC TGC TGG CCT CAC AAG CCC CTG GTT ACT GCA GAT GAA GCT GGG Arg His Cys Trp Pro His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly 240 245 250			950
ATG GAG GCT CTG ACC CCA CCA CCG GCC ACC CAT CTG TCA CCC TTG GAC Met Glu Ala Leu Thr Pro Pro Pro Ala Thr His Leu Ser Pro Leu Asp 255 260 265			998
AGC GCC CAC ACC CTT CTA GCA CCT CCT GAC AGC AGT GAG AAG ATC TGC Ser Ala His Thr Leu Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys 270 275 280			1046
ACC GTC CAG TTG GTG GGT AAC AGC TGG ACC CCT GGC TAC CCC GAG ACC Thr Val Gln Leu Val Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr 285 290 295			1094
CAG GAG GCG CTC TGC CCG CAG GTG ACA TGG TCC TGG GAC CAG TTG CCC Gln Glu Ala Leu Cys Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro 300 305 310 315			1142
AGC AGA GCT CTT GGC CCC GCT GCT GCG CCC ACA CTC TCG CCA GAG TCC Ser Arg Ala Leu Gly Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser 320 325 330			1190
CCA GCC GGC TCG CCA GCC ATG ATG CTG CAG CCG GGC CCG CAG CTC TAC Pro Ala Gly Ser Pro Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr 335 340 345			1238
GAC GTG ATG GAC GCG GTC CCA GCG CGG CGC TGG AAG GAG TTC GTG CGC Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg 350 355 360			1286
ACG CTG GGG CTG CGC GAG GCA GAG ATC GAA GCC GTG GAG GTG GAG ATC Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile 365 370 375			1334
GGC CGC TTC CGA GAC CAG CAG TAC GAG ATG CTC AAG CGC TGG CGC CAG			1382

Gly Arg Phe Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln	
380 385 390 395	
CAG CAG CCC GCG GGC CTC GGA GCC GTT TAC GCG GCC CTG GAG CGC ATG	1430
Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met	
400 405 410	
GGG CTG GAC GGC TGC GTG GAA GAC TTG CGC AGC CGC CTG CAG CGC GGC	1478
Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly	
415 420 425	
CCG TGACACGGCG CCCACTTGCC ACCTAGGCGC TCTGGTGGCC CTTGCAGAAG	1531
Pro	
CCCTAAGTAC GGTACTTAT GCGTGTAGAC ATTTTATGTC ACTTATTAAG CCGCTGGCAC	1591
GGCCCTGCGT AGCAGCACCA GCCGGCCCCA CCCCTGCTCG CCCCTATCGC TCCAGCCAAG	1651
GCGAAGAAGC ACGAACGAAT GTCGAGAGGG GGTGAAGACA TTTCTCAACT TCTCGGCCGG	1711
AGTTTGGCTG AGATCGCGGT ATTAAATCTG TGAAAGAAAA CAAAACAAAA CAAAAAAAAA	1771
AAAAAAAAAA AA	1783

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 428 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg Gly Gln Leu Arg Gly	
1 5 10 15	
Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu Leu Val Leu Leu Gly	
20 25 30	
Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg Cys Asp Cys Ala Gly	
35 40 45	
Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys Arg Gly Cys Pro Ala	
50 55 60	
Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro Cys Gly Asn Ser Thr	
65 70 75 80	
Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala Trp Glu Asn His His	
85 90 95	

Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp Glu Gln Ala Ser Gln	100	105	110
Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp Thr Arg Cys Gly Cys	115	120	125
Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser Gln Cys Val Ser Ser	130	135	140
Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys Gly Ala Leu His Arg	145	150	155
His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr Asp Cys Gly Thr Cys	165	170	175
Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys Val Ser Cys Pro Thr	180	185	190
Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala Ala Val Cys Gly Trp	195	200	205
Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala Gly Leu Val Val Pro	210	215	220
Leu Leu Leu Gly Ala Thr Leu Thr Tyr Thr Tyr Arg His Cys Trp Pro	225	230	235
His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly Met Glu Ala Leu Thr	245	250	255
Pro Pro Pro Ala Thr His Leu Ser Pro Leu Asp Ser Ala His Thr Leu	260	265	270
Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys Thr Val Gln Leu Val	275	280	285
Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr Gln Glu Ala Leu Cys	290	295	300
Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro Ser Arg Ala Leu Gly	305	310	315
Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser Pro Ala Gly Ser Pro	325	330	335
Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr Asp Val Met Asp Ala	340	345	350
Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg Thr Leu Gly Leu Arg	355	360	365
Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile Gly Arg Phe Arg Asp	370	375	380
Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln Gln Gln Pro Ala Gly			

ATG	GAG	CAG	CGG	CCG	CGG	GGC	TGC	GCG	GCG	GTG	GCG	GCG	GCG	CTC	CTC	48
Met	Glu	Gln	Arg	Pro	Arg	Gly	Cys	Ala	Ala	Val	Ala	Ala	Ala	Leu	Leu	
	430					435					440					
CTG	GTG	CTG	CTG	GGG	GCC	CGG	GCC	CAG	GGC	GGC	ACT	CGT	AGC	CCC	AGG	96
Leu	Val	Leu	Leu	Gly	Ala	Arg	Ala	Gln	Gly	Gly	Thr	Arg	Ser	Pro	Arg	
	445				450					455					460	
TGT	GAC	TGT	GCC	GGT	GAC	TTC	CAC	AAG	AAG	ATT	GGT	CTG	TTT	TGT	TGC	144
Cys	Asp	Cys	Ala	Gly	Asp	Phe	His	Lys	Lys	Ile	Gly	Leu	Phe	Cys	Cys	
				465					470					475		
AGA	GGC	TGC	CCA	GCG	GGG	CAC	TAC	CTG	AAG	GCC	CCT	TGC	ACG	GAG	CCC	192
Arg	Gly	Cys	Pro	Ala	Gly	His	Tyr	Leu	Lys	Ala	Pro	Cys	Thr	Glu	Pro	
			480					485					490			
TGC	GGC	AAC	TCC	ACC	TGC	CTT	GTG	TGT	CCC	CAA	GAC	ACC	TTC	TTG	GCC	240
Cys	Gly	Asn	Ser	Thr	Cys	Leu	Val	Cys	Pro	Gln	Asp	Thr	Phe	Leu	Ala	
		495					500					505				
TGG	GAG	AAC	CAC	CAT	AAT	TCT	GAA	TGT	GCC	CGC	TGC	CAG	GCC	TGT	GAT	288
Trp	Glu	Asn	His	His	Asn	Ser	Glu	Cys	Ala	Arg	Cys	Gln	Ala	Cys	Asp	
	510					515					520					
GAG	CAG	GCC	TCC	CAG	GTG	GCG	CTG	GAG	AAC	TGT	TCA	GCA	GTG	GCC	GAC	336
Glu	Gln	Ala	Ser	Gln	Val	Ala	Leu	Glu	Asn	Cys	Ser	Ala	Val	Ala	Asp	
	525				530					535					540	
ACC	CGC	TGT	GGC	TGT	AAG	CCA	GGC	TGG	TTT	GTG	GAG	TGC	CAG	GTC	AGC	384

Thr	Arg	Cys	Gly	Cys	Lys	Pro	Gly	Trp	Phe	Val	Glu	Cys	Gln	Val	Ser	
				545					550					555		
CAA	TGT	GTC	AGC	AGT	TCA	CCC	TTC	TAC	TGC	CAA	CCA	TGC	CTA	GAC	TGC	432
Gln	Cys	Val	Ser	Ser	Ser	Pro	Phe	Tyr	Cys	Gln	Pro	Cys	Leu	Asp	Cys	
			560					565					570			
GGG	GCC	CTG	CAC	CGC	CAC	ACA	CGG	CTA	CTC	TGT	TCC	CGC	AGA	GAT	ACT	480
Gly	Ala	Leu	His	Arg	His	Thr	Arg	Leu	Leu	Cys	Ser	Arg	Arg	Asp	Thr	
			575				580					585				
GAC	TGT	GGG	ACC	TGC	CTG	CCT	GGC	TTC	TAT	GAA	CAT	GGC	GAT	GGC	TGC	528
Asp	Cys	Gly	Thr	Cys	Leu	Pro	Gly	Phe	Tyr	Glu	His	Gly	Asp	Gly	Cys	
		590				595					600					
GTG	TCC	TGC	CCC	ACG	AGC	ACC	CTG	GGG	AGC	TGT	CCA	GAG	CGC	TGT	GCC	576
Val	Ser	Cys	Pro	Thr	Ser	Thr	Leu	Gly	Ser	Cys	Pro	Glu	Arg	Cys	Ala	
605						610				615					620	
GCT	GTC	TGT	GGC	TGG	AGG	CAG	ATG	TTC	TGG	GTC	CAG	GTG	CTC	CTG	GCT	624
Ala	Val	Cys	Gly	Trp	Arg	Gln	Met	Phe	Trp	Val	Gln	Val	Leu	Leu	Ala	
			625					630					635			
GGC	CTT	GTG	GTC	CCC	CTC	CTG	CTT	GGG	GCC	ACC	CTG	ACC	TAC	ACA	TAC	672
Gly	Leu	Val	Val	Pro	Leu	Leu	Leu	Gly	Ala	Thr	Leu	Thr	Tyr	Thr	Tyr	
			640					645					650			
CGC	CAC	TGC	TGG	CCT	CAC	AAG	CCC	CTG	GTT	ACT	GCA	GAT	GAA	GCT	GGG	720
Arg	His	Cys	Trp	Pro	His	Lys	Pro	Leu	Val	Thr	Ala	Asp	Glu	Ala	Gly	
		655					660					665				
ATG	GAG	GCT	CTG	ACC	CCA	CCA	CCG	GCC	ACC	CAT	CTG	TCA	CCC	TTG	GAC	768
Met	Glu	Ala	Leu	Thr	Pro	Pro	Pro	Ala	Thr	His	Leu	Ser	Pro	Leu	Asp	
		670				675					680					
AGC	GCC	CAC	ACC	CTT	CTA	GCA	CCT	CCT	GAC	AGC	AGT	GAG	AAG	ATC	TGC	816
Ser	Ala	His	Thr	Leu	Leu	Ala	Pro	Pro	Asp	Ser	Ser	Glu	Lys	Ile	Cys	
685						690				695					700	
ACC	GTC	CAG	TTG	GTG	GGT	AAC	AGC	TGG	ACC	CCT	GGC	TAC	CCC	GAG	ACC	864
Thr	Val	Gln	Leu	Val	Gly	Asn	Ser	Trp	Thr	Pro	Gly	Tyr	Pro	Glu	Thr	
			705					710						715		
CAG	GAG	GCG	CTC	TGC	CCG	CAG	GTG	ACA	TGG	TCC	TGG	GAC	CAG	TTG	CCC	912
Gln	Glu	Ala	Leu	Cys	Pro	Gln	Val	Thr	Trp	Ser	Trp	Asp	Gln	Leu	Pro	
			720					725					730			
AGC	AGA	GCT	CTT	GGC	CCC	GCT	GCT	GCG	CCC	ACA	CTC	TCG	CCA	GAG	TCC	960
Ser	Arg	Ala	Leu	Gly	Pro	Ala	Ala	Ala	Pro	Thr	Leu	Ser	Pro	Glu	Ser	
		735					740					745				
CCA	GCC	GGC	TCG	CCA	GCC	ATG	ATG	CTG	CAG	CCG	GGC	CCG	CAG	CTC	TAC	1008
Pro	Ala	Gly	Ser	Pro	Ala	Met	Met	Leu	Gln	Pro	Gly	Pro	Gln	Leu	Tyr	
		750				755					760					

GAC GTG ATG GAC GCG GTC CCA GCG CGG CGC TGG AAG GAG TTC GTG CGC	1056
Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg	
765 770 775 780	
ACG CTG GGG CTG CGC GAG GCA GAG ATC GAA GCC GTG GAG GTG GAG ATC	1104
Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile	
785 790 795	
GGC CGC TTC CGA GAC CAG CAG TAC GAG ATG CTC AAG CGC TGG CGC CAG	1152
Gly Arg Phe Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln	
800 805 810	
CAG CAG CCC GCG GGC CTC GGA GCC GTT TAC GCG GCC CTG GAG CGC ATG	1200
Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met	
815 820 825	
GGG CTG GAC GGC TGC GTG GAA GAC TTG CGC AGC CGC CTG CAG CGC GGC	1248
Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly	
830 835 840	
CCG TGA	1254
Pro	
845	

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu Gln Arg Pro Arg Gly Cys Ala Ala Val Ala Ala Ala Leu Leu	
1 5 10 15	
Leu Val Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg	
20 25 30	
Cys Asp Cys Ala Gly Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys	
35 40 45	
Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro	
50 55 60	
Cys Gly Asn Ser Thr Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala	
65 70 75 80	
Trp Glu Asn His His Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp	
85 90 95	
Glu Gln Ala Ser Gln Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp	

100	105	110
Thr Arg Cys Gly Cys Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser		
115	120	125
Gln Cys Val Ser Ser Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys		
130	135	140
Gly Ala Leu His Arg His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr		
145	150	155
Asp Cys Gly Thr Cys Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys		
165	170	175
Val Ser Cys Pro Thr Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala		
180	185	190
Ala Val Cys Gly Trp Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala		
195	200	205
Gly Leu Val Val Pro Leu Leu Leu Gly Ala Thr Leu Thr Tyr Thr Tyr		
210	215	220
Arg His Cys Trp Pro His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly		
225	230	235
Met Glu Ala Leu Thr Pro Pro Pro Ala Thr His Leu Ser Pro Leu Asp		
245	250	255
Ser Ala His Thr Leu Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys		
260	265	270
Thr Val Gln Leu Val Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr		
275	280	285
Gln Glu Ala Leu Cys Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro		
290	295	300
Ser Arg Ala Leu Gly Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser		
305	310	315
Pro Ala Gly Ser Pro Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr		
325	330	335
Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg		
340	345	350
Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile		
355	360	365
Gly Arg Phe Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln		
370	375	380
Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met		
385	390	395
		400

Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly
 405 410 415

Pro

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 455 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Gly	Leu	Ser	Thr	Val	Pro	Asp	Leu	Leu	Leu	Pro	Leu	Val	Leu	Leu	1	5	10	15
Glu	Leu	Leu	Val	Gly	Ile	Tyr	Pro	Ser	Gly	Val	Ile	Gly	Leu	Val	Pro	20	25	30	
His	Leu	Gly	Asp	Arg	Glu	Lys	Arg	Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	35	40	45	
Tyr	Ile	His	Pro	Gln	Asn	Asn	Ser	Ile	Cys	Cys	Thr	Lys	Cys	His	Lys	50	55	60	
Gly	Thr	Tyr	Leu	Tyr	Asn	Asp	Cys	Pro	Gly	Pro	Gly	Gln	Asp	Thr	Asp	65	70	75	80
Cys	Arg	Glu	Cys	Glu	Ser	Gly	Ser	Phe	Thr	Ala	Ser	Glu	Asn	His	Leu	85	90	95	
Arg	His	Cys	Leu	Ser	Cys	Ser	Lys	Cys	Arg	Lys	Glu	Met	Gly	Gln	Val	100	105	110	
Glu	Ile	Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	Cys	Gly	Cys	Arg	115	120	125	
Lys	Asn	Gln	Tyr	Arg	His	Tyr	Trp	Ser	Glu	Asn	Leu	Phe	Gln	Cys	Phe	130	135	140	
Asn	Cys	Ser	Leu	Cys	Leu	Asn	Gly	Thr	Val	His	Leu	Ser	Cys	Gln	Glu	145	150	155	160
Lys	Gln	Asn	Thr	Val	Cys	Thr	Cys	His	Ala	Gly	Phe	Phe	Leu	Arg	Glu	165	170	175	
Asn	Glu	Cys	Val	Ser	Cys	Ser	Asn	Cys	Lys	Lys	Ser	Leu	Glu	Cys	Thr				

180	185	190
Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser		
195	200	205
Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu		
210	215	220
Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys		
225	230	235
Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu		
245	250	255
Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser		
260	265	270
Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val		
275	280	285
Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys		
290	295	300
Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly		
305	310	315
Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn		
325	330	335
Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp		
340	345	350
Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro		
355	360	365
Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu		
370	375	380
Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln		
385	390	395
Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala		
405	410	415
Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly		
420	425	430
Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro		
435	440	445
Pro Ala Pro Ser Leu Leu Arg		
450	455	

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 335 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Leu	Gly	Ile	Trp	Thr	Leu	Leu	Pro	Leu	Val	Leu	Thr	Ser	Val	Ala	
1				5					10					15		
Arg	Leu	Ser	Ser	Lys	Ser	Val	Asn	Ala	Gln	Val	Thr	Asp	Ile	Asn	Ser	
		20					25						30			
Lys	Gly	Leu	Glu	Leu	Arg	Lys	Thr	Val	Thr	Thr	Val	Glu	Thr	Gln	Asn	
	35					40						45				
Leu	Glu	Gly	Leu	His	His	Asp	Gly	Gln	Phe	Cys	His	Lys	Pro	Cys	Pro	
	50					55					60					
Pro	Gly	Glu	Arg	Lys	Ala	Arg	Asp	Cys	Thr	Val	Asn	Gly	Asp	Glu	Pro	
65				70					75					80		
Asp	Cys	Val	Pro	Cys	Gln	Glu	Gly	Lys	Glu	Tyr	Thr	Asp	Lys	Ala	His	
			85					90						95		
Phe	Ser	Ser	Lys	Cys	Arg	Arg	Cys	Arg	Leu	Cys	Asp	Glu	Gly	His	Gly	
			100					105						110		
Leu	Glu	Val	Glu	Ile	Asn	Cys	Thr	Arg	Thr	Gln	Asn	Thr	Lys	Cys	Arg	
		115					120					125				
Cys	Lys	Pro	Asn	Phe	Phe	Gln	Asn	Ser	Thr	Val	Cys	Glu	His	Cys	Asp	
	130					135					140					
Pro	Cys	Thr	Lys	Cys	Glu	His	Gly	Ile	Ile	Lys	Glu	Cys	Thr	Leu	Thr	
145				150						155				160		
Ser	Asn	Thr	Lys	Cys	Lys	Glu	Glu	Gly	Ser	Arg	Ser	Asn	Leu	Gly	Trp	
			165						170					175		
Leu	Cys	Leu	Leu	Leu	Leu	Pro	Ile	Pro	Leu	Ile	Val	Trp	Val	Lys	Arg	
		180					185						190			
Lys	Glu	Val	Gln	Lys	Thr	Cys	Arg	Lys	His	Arg	Lys	Glu	Asn	Gln	Gly	
	195					200						205				
Ser	His	Glu	Ser	Pro	Thr	Leu	Asn	Pro	Glu	Thr	Val	Ala	Ile	Asn	Leu	
	210					215						220				

Ser	Asp	Val	Asp	Leu	Ser	Lys	Tyr	Ile	Thr	Thr	Ile	Ala	Gly	Val	Met
225					230					235					240
Thr	Leu	Ser	Gln	Val	Lys	Gly	Phe	Val	Arg	Lys	Asn	Gly	Val	Asn	Glu
			245						250					255	
Ala	Lys	Ile	Asp	Glu	Ile	Lys	Asn	Asp	Asn	Val	Gln	Asp	Thr	Ala	Glu
			260					265					270		
Gln	Lys	Val	Gln	Leu	Leu	Arg	Asn	Trp	His	Gln	Leu	His	Gly	Lys	Lys
		275					280					285			
Glu	Ala	Tyr	Asp	Thr	Leu	Ile	Lys	Asp	Leu	Lys	Lys	Ala	Asn	Leu	Cys
	290					295					300				
Thr	Leu	Ala	Glu	Lys	Ile	Gln	Thr	Ile	Ile	Leu	Lys	Asp	Ile	Thr	Ser
305					310					315					320
Asp	Ser	Glu	Asn	Ser	Asn	Phe	Arg	Asn	Glu	Ile	Gln	Ser	Leu	Val	
			325						330					335	

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGCCATGGG GGCCCGGCGG CAG

23

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGAAGCTTC TAGGACCCAG AACATCTGCC

30

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCGGATCCG CCATCATGGA GGAGACGCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGGATCCG CCATCATGGA GCAGCGGCCG CGG

33

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 54 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCGTCTAGAT CAAAGCGTAG TCTGGGACGT CGTATGGGTA CGGGCCGCGC TGCA

54

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCGGATCCG CCATCATGGA GGAGACGCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGCGGATCCG CCATCATGGA GCAGCGGCCG CGG

33

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGCGGATCCT CACGGGCCGC GCTGCA

26

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGCGGATCCG CCATCATGGA GGAGACGCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGCGGATCCG CCATCATGGA GCAGCGGCCG CGG

33

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGAGATCTA GTCTGGACCC AGAACATCTG CCTCC

35

What Is Claimed Is:

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

5 (a) a nucleotide sequence encoding the full-length death domain containing receptor polypeptide (DR3-V1) having the complete amino acid sequence in Figure 1 (SEQ ID NO:2);

(b) nucleotide sequence encoding the full-length death domain containing receptor (DR3) polypeptide having the complete amino acid sequence
10 in Figure 2 (SEQ ID NO:4), including the predicted leader sequence;

(c) a nucleotide sequence encoding the DR3-V1 polypeptide having the amino acid sequence at positions from about 36 to about 428 in Figure 1 (SEQ ID NO:2);

(d) a nucleotide sequence encoding the full-length DR3-V1
15 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97456;

(e) a nucleotide sequence encoding the full-length DR3 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. _____;

20 (f) a nucleotide sequence encoding the mature DR3-V1 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456;

(g) a nucleotide sequence encoding the mature DR3 polypeptide having the amino acid sequence encoded by the cDNA clone
25 contained in ATCC Deposit No. _____;

(h) a nucleotide sequence that encodes the DR3 extracellular domain;

(i) a nucleotide sequence that encodes the DR3 transmembrane domain;

(j) a nucleotide sequence that encodes the DR3 intracellular domain;

(k) a nucleotide sequence that encodes the DR3 death domain; and

5 (l) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k)

2. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence in Figure 1 (SEQ ID NO:1).

10 3. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the death domain containing receptor polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2).

15 4. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the mature death domain containing receptor polypeptide having the amino acid sequence in Figure 1 (SEQ ID NO:2).

5. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 97456.

20 6. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence in Figure 2 (SEQ ID NO:3).

25 7. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the death domain containing receptor polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2).

8. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 2 (SEQ ID NO:3) encoding the mature death domain containing receptor polypeptide having the amino acid sequence in Figure 2 (SEQ ID NO:4).

5 9. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. _____.

10 10. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the death domain containing receptor polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456.

15 11. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the mature death domain containing receptor polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456.

12. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the death domain containing receptor polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. _____.

20 13. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the mature death domain containing receptor polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. _____.

14. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j), (k) or (l) of claim 1 wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

15. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a death domain containing receptor polypeptide having an amino acid sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k) of claim 1.

16. The isolated nucleic acid molecule of claim 15, which encodes an epitope-bearing portion of a death domain containing receptor polypeptide selected from the group consisting of: a polypeptide comprising amino acid residues from about 1 to about 22 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 33 to about 56 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 59 to about 82 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 95 to about 112 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 122 to about 133 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 161 to about 177 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 179 to about 190 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 196 to about 205 in Figure 1 (SEQ ID NO:2).

17. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

18. A recombinant vector produced by the method of claim 17.

19. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 18 into a host cell.

20. A recombinant host cell produced by the method of claim 19.

21. A recombinant method for producing a death domain containing receptor polypeptide, comprising culturing the recombinant host cell of claim 20 under conditions such that said polypeptide is expressed and recovering said polypeptide.

22. An isolated death domain containing receptor polypeptide having an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the amino acid sequence of the DR3-V1 polypeptide having the complete 428 amino acid sequence, including the leader sequence shown in Figure 1 (SEQ ID NO:2);

(b) the amino acid sequence of the DR3 polypeptide having the complete 417 amino acid sequence, including the leader sequence, shown in Figure 2 (SEQ ID NO:4);

(c) the amino acid sequence of the DR3-V1 polypeptide having the amino acid sequence at positions from about 36 to about 428 in Figure 1 (SEQ ID NO:2);

(d) the amino acid sequence of the DR3-V1 polypeptide having the complete amino acid sequence, including the leader, encoded by the cDNA clone contained in ATCC Deposit No. 97456;

(e) the amino acid sequence of the DR3 polypeptide having the complete amino acid sequence, including the leader, encoded by the cDNA clone contained in ATCC Deposit No. _____;

(f) the amino acid sequence of the mature DR3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97456;

5 (g) the amino acid sequence encoding the mature DR3 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. _____;

(h) the amino acid sequence of the DR3 extracellular domain;

(i) the amino acid sequence of the DR3 transmembrane domain;

10 (j) the amino acid sequence of the DR3 intracellular domain;

(k) the amino acid sequence of the DR3 death domain; and

(l) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k).

15 23. An isolated antibody that binds specifically to a death domain containing receptor polypeptide of claim 22.

24. A method of treating diseases and disorders associated with the inhibition of apoptosis comprising administering an effective amount of the polypeptide as claimed in claim 22, or an agonist thereof to a patient in need thereof.

20 25. A method of treating diseases and disorders associated with increased apoptosis comprising administering to a patient in need thereof an effective amount of an antagonist of the polypeptide as claimed in claim 22 to a patient in need thereof.

25 26. A method of treating inflammatory diseases and disorders comprising administering to a patient in need thereof an effective amount of an antagonist of the polypeptide as claimed in claim 22.

10 30 50
 CATGGGTGGGGTGGGGCGCTGCTGGATTCTGCTCTGGTGGAGGGGAAACTTGTGAGG
 70 90 110
 GGCTGGTAAGCGCCCCCTCCGAAGCCTGGTGTGTGCGCGGGGGGAAGGAAGTTAGTTTCC
 130 150 170
 TCTCCACCCATGGGCACCCCTTCTGCCCGGGGCTGGGAAGTGGGCTGCTCTGTGGGCAA
 190 210 230
 ATGCTGGGGCCTCTGAAATGGAGGAGACGCAGCAGGGAGAGGCCCCACGTGGGCAGCTGC
 M E E T O O G E A P R G O L R
 250 270 290
 GCGGAGAGTCAAGCAGCACCTGTCCCCCAGGCGCTCCTCCTGGTGTGCTGGGGGCCCCGGG
 G E S A A P V P O A L L L V L L G A R A
 310 330 350
 CCCAGGGCGGCACTCGTAGCCCCAGGTGTGACTGTGCCGGTGACTTCCACAAGAAGATTG
 Q G G T R S P R C D C A G D F H K K I G
 370 390 410
 GTCTGTTTTGTGTGAGAGGCTGCCAGCGGGGCACTACCTGAAGGCCCTTGCACGGAGC
 L F C C R G C P A G H Y L K A P C T E P
 430 450 470
 CCTGCGGCACTCCACCTGCCTTGTGTGTCCCCAAGACACCTTCTTGGCCTGGGAGAACC
 C G N S T C L V C P Q D T F L A W E N H
 490 510 530
 ACCATAATCTGAATGTGCCCGCTGCCAGGCCTGTGATGAGCAGGCCTCCAGGTGGCGC
 H N S E C A R C Q A C D E Q A S Q V A L
 550 570 590
 TGGAGAACTGTTTCAAGAGTGGCCGACACCCGCTGTGGCTGTAAGCCAGGCTGGTTTGTGG
 E N C S A V A D T R C G C K P G W F V E
 610 630 650
 AGTGCCAGGTCAAGCAATGTGTCAAGCAGTTTACCCTTCTACTGCCAACCATGCCTAGACT
 C Q V S Q C V S S S P F Y C Q P C L D C
 670 690 710
 GCGGGGCCCTGCACCGCCAACACGGCTACTCTGTTCCCGCAGAGATACTGACTGTGGGA
 G A L H R H T R L L C S R R D T D C G T
 730 750 770
 CCTGCCTGCCTGGCTTCTATGAACATGGCGATGGCTGCGTGTCTGCCCCACGAGCACCC
 C L P G F Y E H G D G C V S C P T S T L
 790 810 830
 TGGGGAGCTGTCCATAGCGCTGTGCCGCTGTCTGTGGCTGGAGGCAGATGTTCTGGGTCC
 G S C P E R C A A V C G W R Q M F W V Q
 850 870 890
 AGGTGCTCCTGGCTGGCCTTGTGGTCCCCCTCCTGCTGGGGGCCACCCCTGACCTACACAT
 V L L A G L V V P L L L G A T L T Y T Y
 910 930 950
 ACCGCCACTGCTGGCCTCACAAGCCCCTGGTTACTGCAGATGAAGCTGGGATGGAGGCTC
 R H C W P H K P L V T A D E A G M E A L

FIGURE 1A

970 990 1010
 TGACCCACCCACCGGCCACCCATCTGTACCCCTTGGACAGCGCCACACCCCTTCTAGCAC
 T P P P A T H L S P L D S A H T L L A P

1030 1050 1070
 CTCCTGACAGCAGTGAGAAGATCTGCACCGTCCAGTTGGTGGGTAAACAGCTGGACCCCTG
 P D S S E K I C T V Q L V G N S W T P G

1090 1110 1130
 GCTACCCCGAGACCCAGGAGGCGCTCTGCGCCGACAGGTGACATGGTCCCTGGGACCACTTGC
 Y P E T Q E A L C P Q V T W S W D Q L P

1150 1170 1190
 CCAGCAGAGCTCTTGGCCCCGCTGCTGCGCCACACTCTCGCCAGAGTCCCCAGCCGGCT
 S R A L G P A A A P T L S P E S P A G S

1210 1230 1250
 CGCCAGCATGATGCTGCAGCCGGGCGCCAGCTCTACGACGTGATGGACGCGGTCCAG
 P A M M L Q P G P Q L Y D V M D A V P A

1270 1290 1310
 CGCGGCGCTGGAAGGAGTTCGTGCGCAGCTGGGGCTGCGGAGGCGAGATCGAAGCCG
R R W K E F V R T L G L R E A E I E A V

1330 1350 1370
 TGGAGGTGGAGATCGGCCGCTTCCGAGACCAGCAGTACGAGATGCTCAAGCGCTGGCGCC
E V E I G R F R D O O Y E M L K R W R O

1390 1410 1430
 AGCAGCAGCCCGGGGCTCGGAGCCGTTTACGCGGCCCTGGAGCGCATGGGGCTGGACG
O O P A G L G A V Y A A L E R M G L D G

1450 1470 1490
 GCTGCGTGGAAGACTTGCGCAGCCGCTGCAGCGCGGCCGCTGACACGGCGCCCACTTGC
C V E D L R S R L Q R G P *

1510 1530 1550
 CACCTAGGCGCTCTGGTGGCCCTTGACAGAAGCCCTAAGTACGGTTACTTATGCGGTGAGA

1570 1590 1610
 CATTTTATGTCACTTATTAAGCCGCTGGCACGGCCCTGCGTAGCAGCACCAGCCGGCCCC

1630 1650 1670
 ACCCCTGCTCGCCCCTATCGCTCCAGCCAAGGCGAAGAAGCACGAACGAATGTCGAGAGG

1690 1710 1730
 GGGTGAAGACATTTCTCAACTTCTCGGCCGAGTTTGGCTGAGATCGCGGTATTAAATCT

1750 1770
 GTGAAAGAAAACAAAACAAAACAAAAAAAAAAAAAAAAAAAA

FIGURE 1B

1 ATGGAGCAGC GCCCGCGGGG CTGCGCGGGG GTGGCGGGGG CGCTCCTCCT GGTGCTGCTG
 M E Q R P R G C A A V A A A L L L V L L
 61 GGGGCCCCGG CCCAGGGCGG CACTCGTAGC CCCAGGTGTG ACTGTGCCGG TGACTTCCAC
 G A R A Q G G T R S P R C D C A G D F H
 121 AAGAAGATTG GTCTGTTTTG TTGCAGAGGC TGCCCGAGCG GGCCTACCT GAAGGCCCCCT
 K K I G L F C C R G C P A G H Y L K A P
 181 TGCACGGAGC CCTGCGGCAA CTCCACCTGC CTTGTGTGTC CCCAAGACAC CTTCTTGGCC
 C T E P C G N S T C L V C P Q D T F L A
 241 TGGGAGAACC ACCATAATTC TGAATGTGCC CGCTGCCAGG CCTGTGATGA GCAGGCCTCC
 W E N H H N S E C A R C Q A C D E Q A S
 301 CAGGTGGCGC TGGAGAACTG TTCAGCAGTG GCCGACACCC GCTGTGGCTG TAAGCCAGGC
 Q V A L E N C S A V A D T R C G C K P G
 361 TGGTTTGTGG AGTGCCAGGT CAGCCAATGT GTCAGCAGTT CACCCTTCTA CTGCCAACCA
 W F V E C Q V S Q C V S S S P F Y C Q P
 421 TGCCTAGACT GCGGGGCCCT GCACCGCCAC ACACGGCTAC TCTGTTCCCG CAGAGATACT
 C L D C G A L H R H T R L L C S R R D T
 481 GACTGTGGGA CCTGCCTGCC TGGCTTCTAT GAACATGGCG ATGGCTGCGT GTCCTGCCCC
 D C G T C L P G F Y E H G D G C V S C P
 541 ACGAGCACCC TGGGAGCTG TCCAGAGCGC TGTGCCGCTG TCTGTGGCTG GAGGCAGATG
 T S T L G S C P E R C A A V C G W R Q M
 601 TTCTGGGTCC AGGTGCTCCT GGCTGGCCTT GTGGTCCCCC TCCTGCTTGG GGCCACCCTG
 F W V Q V L L A G L V V P L L L G A T L
 661 ACCTACACAT ACCGCCACTG CTGGCCTCAC AAGCCCCTGG TTACTGCAGA TGAAGCTGGG
 T Y T Y R H C W P H K P L V T A D E A G
 721 ATGGAGGCTC TGACCCACCC ACCGGCCACC CATCTGTCAC CCTTGGACAG CGCCACACC
 M E A L T P P P A T H L S P L D S A H T
 781 CTTCTAGCAC CTCCTGACAG CAGTGAGAAG ATCTGCACCG TCCAGTTGGT GGGTAACAGC
 L L A P P D S S E K I C T V Q L V G N S
 841 TGGACCCCTG GCTACCCCGA GACCCAGGAG GCGCTCTGCC CGCAGGTGAC ATGGTCTCTG
 W T P G Y F E T Q E A L C P Q V T W S W
 901 GACCAGTTGC CCAGCAGAGC TCTTGGCCCC GCTGCTGCGC CCACACTCTC GCCAGAGTCC
 D Q L P S R A L G P A A A P T L S P E S
 961 CCAGCCGGCT CGCCAGCCAT GATGCTGCAG CCGGGGCGGC AGCTCTACGA CGTGATGGAC
 P A G S P A M M L Q P G P Q L Y D V M D
 1021 GCGGTCCAG CGCGGCGCTG GAAGGAGTTC GTGCGCACGC TGGGGCTGCG CGAGGCAGAG
 A V P A R R W K E F V R T L G L R E A E
 1081 ATCGAAGCCG TGGAGGTGGA GATCGGCCGC TTCCGAGACC AGCAGTACGA GATGCTCAAG
 I E A V E V E I G R F R D Q Q Y E M L K
 1141 CGCTGGCGCC AGCAGCAGCC CGCGGGCCTC GGAGCCGTTT ACGCGGCCCT GGAGCGCATG
 R W R Q Q Q P A G L G A V Y A A L E R M
 1201 GGGCTGGACG GCTGCGTGGA AGACTTGGCG AGCGCGCTGC AGGGCGGCCC GTGA
 G L D G C V E D L R S R L Q R G P

FIGURE 2

Consensus #1 M

DDCR M E E T Q Q G E A P R G Q L R G E S A A P V P Q A L L L V L 30
 TNFR1 M G L S T V P D L L L P L V L L E L L V G I Y P S G V I G L 30
 FAS M - L G I W T L L L P L V L T S V A R L S S K S V N A Q V T D 29

Consensus #1 C

DDCR L G A R A Q G G T R S P R C D C A G D F H - - K K I G L E C 58
 TNFR1 V P H L G D R E K R D S V C P Q G K Y I H - - P Q N N S I C 58
 FAS I N S K G L E L R K T V T V E T Q N L E G L H H D G Q E C 59

Consensus #1 . . . C . . G C C . . C . . .

DDCR C R G C P A G H Y L K A P C T E P C G N S T C L V C P Q D T 88
 TNFR1 C T K C H K G T Y L Y N D C P G P G Q D T D C R E C E S G S 88
 FAS H K P C P P G E R K A R D C T V N G D E P D C V P C Q E G K 89

Consensus #1 H C . . C . . C C

DDCR F L A W E N H H N S E C A R C Q A C D E Q A S Q V A L E N C 118
 TNFR1 F T A S E N H L R - H C L S C S K C R K E M G O V E I S S C 117
 FAS E Y T D K A H F S S K C R R C R L C D E G H G L E V E I N C 119

Consensus #1 T . C . C

DDCR S A V A D T R C G C K P G W F V E C - - - Q V S Q C V S S S 145
 TNFR1 T V D R D T V C G C R K N Q Y R H Y W S E N L F O C - - - 144
 FAS T R T Q N T K C R C K P N F F Q N - - - - - - - - - 137

Consensus #1 C

DDCR P F Y C Q P C L D C G A L H R H T R L L C S R R D T D C G T 175
 TNFR1 - F N C S L C L N - G T V H - - - - L S C Q E K Q N T V C T 167
 FAS - - - S T V C E H C D P - - - - - - - - - - C T K 148

Consensus #1 C . . G C

DDCR C L P G F Y E H G D G C V S C P T S T L G - S C P E R C - - 203
 TNFR1 C H A G F F L R E N E C V S C S N C K K S L E C T K L C L P 197
 FAS C E H G I I - - - K E C - - - - - - - - - - T L T S N T K C - - 166

Consensus #1 L

DDCR - - - - - A A V C G W R Q M F W V Q V L L A G L V V P L 225
 TNFR1 Q I E N V K G T E D S G T T V L L P L V I F F G L C L L S L 227
 FAS - - - - - K E E G S R S N L G W L C L L L - - L P I P L 186

Consensus #1

DDCR L L G G T L D L H I P P L L A H K P L V T A D E A G M E A L 255
 TNFR1 L F I G - L M Y R Y Q R W K S K L Y S I V C G K S T P E K E 256
 FAS I V - - - - - - - - - W V K R K E V - - - Q K T C R K H R 203

Consensus #1 G . . . P

DDCR N P P P G T H L S P L D S A H T L L A P P D S S E K I C T V 285
 TNFR1 G E L E G T T T K P L A P N P S F S P T P G F T P T L G F S 286
 FAS K E N Q G S H E S P - - - - - - - - - - - - - - - 214

FIGURE 3

Consensus #1

DDCR Q L V G N S W **T** P G Y P E **T** Q E A L **C** P Q V T W S W D Q L - 315
 TNFR1 P V P S S T F **T** S S S T Y **T** P G D - **C** P N F A A P R R E V A 315
 FAS - - - - - 214

Consensus #1 L

DDCR - **P** S R A L G **P** A A **A** P **T** L S **P** E S **P** A G S - - - - - 336
 TNFR1 P **P** Y Q G A D **P** I L A **T** A L A S D **P** I P N **P** L Q K W E D S A 345
 FAS - - - - - **T** L N **P** E T V A I N **L** S - - - - - 226

Consensus #1 K . F V

DDCR - - - P A M M L O P G P Q **L** Y D V M D A V P A R R W K E F V 362
 TNFR1 H K P Q S L **D** T D D P A T L Y A V V E N V P L R W K E F V 375
 FAS - - - - - **D** V D L S K Y I T T I A G V M T L S Q V **K** G F V 249

Consensus #1 R . . G I L .

DDCR **R** T L G L R E A E I E A V E V E I G R - F R D O Q Y E M L K 391
 TNFR1 **R** R L G L S D H E I D R L E L Q N G R C L R E A Q Y S M L A 405
 FAS **R** K N G V N E A K I D E I K N D N V Q D T A E Q K V Q L L R 279

Consensus #1 . W A L E

DDCR R **W** R Q Q Q P - - - **A** G L G A V Y A A L E R M G L D G C V E 418
 TNFR1 T **W** R R R T P R R E A T L E L L G R V L R D M D L L G C L E 435
 FAS N **W** H Q L H G K K E A - Y D T L I K D L K K A N L C T L A E 308

Consensus #1

DDCR **D** L - - - - - R **S** R L Q R G P 428
 TNFR1 **D** I E E A L - - - - - C G P A A L P P A P S L L R 455
 FAS **K** I Q T I I L K D I T S D S E N S N F R N E I Q S L V 335

Consensus 'Consensus #1': When all match the residue of the Consensus show the residue of the Consensus, otherwise show '.'.

Decoration 'Decoration #1': Shade (with solid black) residues that match the Consensus exactly.

FIGURE 3 (CONT'D)

RAW SEQUENCE LISTING PATENT APPLICATION US/60/028,711

DATE: 11/29/96
TIME: 11:24:57

INPUT SET: S14126.raw

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47      (A) LENGTH: 1783 base pairs
48      (B) TYPE: nucleic acid
49      (C) STRANDEDNESS: double
50      (D) TOPOLOGY: both
51
52      (ii) MOLECULE TYPE: cDNA
53
54
55      (ix) FEATURE:
56          (A) NAME/KEY: CDS
57          (B) LOCATION: 198..1481
58
59
60      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
61
62      CATGGGTGGG GGTGGGGGCG CTGCTGGATT CCTGCTCTGG TGGAGGGGAA ACTTGTGAGG      60
63
64      GGCTGGTAAG CGCCCCCTCC GAAGCCTGGT GTGTGCGCGG GGGGAAGGAA GTTAGTTTCC      120
65
66      TCTCCACCCA TGGGCACCCC TTCTGCCCGG GGCCTGGGAA GTGGGCTGCT CTGTGGGCAA      180
67
68      ATGCTGGGGC CTCTGAA ATG GAG GAG ACG CAG CAG GGA GAG GCC CCA CGT      230
69      Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg
70      1 5 10
71
72      GGG CAG CTG CGC GGA GAG TCA GCA GCA CCT GTC CCC CAG GCG CTC CTC      278
73      Gly Gln Leu Arg Gly Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu
74      15 20 25
75
76      CTG GTG CTG CTG GGG GCC CGG GCC CAG GGC GGC ACT CGT AGC CCC AGG      326
77      Leu Val Leu Leu Gly Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg
78      30 35 40
79
80      TGT GAC TGT GCC GGT GAC TTC CAC AAG AAG ATT GGT CTG TTT TGT TGC      374
81      Cys Asp Cys Ala Gly Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys
82      45 50 55
83
84      AGA GGC TGC CCA GCG GGG CAC TAC CTG AAG GCC CCT TGC ACG GAG CCC      422
85      Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro
86      60 65 70 75
87
88      TGC GGC AAC TCC ACC TGC CTT GTG TGT CCC CAA GAC ACC TTC TTG GCC      470
89      Cys Gly Asn Ser Thr Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala
90      80 85 90
91
92      TGG GAG AAC CAC CAT AAT TCT GAA TGT GCC CGC TGC CAG GCC TGT GAT      518
93      Trp Glu Asn His His Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp
94      95 100 105
95
96      GAG CAG GCC TCC CAG GTG GCG CTG GAG AAC TGT TCA GCA GTG GCC GAC      566
97      Glu Gln Ala Ser Gln Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp
98      110 115 120
99

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INPUT SET: S14126.raw

100	ACC CGC TGT GGC TGT AAG CCA GGC TGG TTT GTG GAG TGC CAG GTC AGC	614
101	Thr Arg Cys Gly Cys Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser	
102	125 130 135	
103		
104	CAA TGT GTC AGC AGT TCA CCC TTC TAC TGC CAA CCA TGC CTA GAC TGC	662
105	Gln Cys Val Ser Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys	
106	140 145 150 155	
107		
108	GGG GCC CTG CAC CGC CAC ACA CGG CTA CTC TGT TCC CGC AGA GAT ACT	710
109	Gly Ala Leu His Arg His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr	
110	160 165 170	
111		
112	GAC TGT GGG ACC TGC CTG CCT GGC TTC TAT GAA CAT GGC GAT GGC TGC	758
113	Asp Cys Gly Thr Cys Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys	
114	175 180 185	
115		
116	GTG TCC TGC CCC ACG AGC ACC CTG GGG AGC TGT CCA GAG CGC TGT GCC	806
117	Val Ser Cys Pro Thr Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala	
118	190 195 200	
119		
120	GCT GTC TGT GGC TGG AGG CAG ATG TTC TGG GTC CAG GTG CTC CTG GCT	854
121	Ala Val Cys Gly Trp Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala	
122	205 210 215	
123		
124	GGC CTT GTG GTC CCC CTC CTG CTT GGG GCC ACC CTG ACC TAC ACA TAC	902
125	Gly Leu Val Val Pro Leu Leu Leu Gly Ala Thr Leu Thr Tyr Thr Tyr	
126	220 225 230 235	
127		
128	CGC CAC TGC TGG CCT CAC AAG CCC CTG GTT ACT GCA GAT GAA GCT GGG	950
129	Arg His Cys Trp Pro His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly	
130	240 245 250	
131		
132	ATG GAG GCT CTG ACC CCA CCA CCG GCC ACC CAT CTG TCA CCC TTG GAC	998
133	Met Glu Ala Leu Thr Pro Pro Pro Ala Thr His Leu Ser Pro Leu Asp	
134	255 260 265	
135		
136	AGC GCC CAC ACC CTT CTA GCA CCT CCT GAC AGC AGT GAG AAG ATC TGC	1046
137	Ser Ala His Thr Leu Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys	
138	270 275 280	
139		
140	ACC GTC CAG TTG GTG GGT AAC AGC TGG ACC CCT GGC TAC CCC GAG ACC	1094
141	Thr Val Gln Leu Val Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr	
142	285 290 295	
143		
144	CAG GAG GCG CTC TGC CCG CAG GTG ACA TGG TCC TGG GAC CAG TTG CCC	1142
145	Gln Glu Ala Leu Cys Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro	
146	300 305 310 315	
147		
148	AGC AGA GCT CTT GGC CCC GCT GCT GCG CCC ACA CTC TCG CCA GAG TCC	1190
149	Ser Arg Ala Leu Gly Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser	
150	320 325 330	
151		
152	CCA GCC GGC TCG CCA GCC ATG ATG CTG CAG CCG GGC CCG CAG CTC TAC	1238

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153  Pro Ala Gly Ser Pro Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr
154                      335                      340                      345
155
156  GAC GTG ATG GAC GCG GTC CCA GCG CGG CGC TGG AAG GAG TTC GTG CGC      1286
157  Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg
158                      350                      355                      360
159
160  ACG CTG GGG CTG CGC GAG GCA GAG ATC GAA GCC GTG GAG GTG GAG ATC      1334
161  Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile
162                      365                      370                      375
163
164  GGC CGC TTC CGA GAC CAG CAG TAC GAG ATG CTC AAG CGC TGG CGC CAG      1382
165  Gly Arg Phe Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln
166  380                      385                      390                      395
167
168  CAG CAG CCC GCG GGC CTC GGA GCC GTT TAC GCG GCC CTG GAG CGC ATG      1430
169  Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met
170                      400                      405                      410
171
172  GGG CTG GAC GGC TGC GTG GAA GAC TTG CGC AGC CGC CTG CAG CGC GGC      1478
173  Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly
174                      415                      420                      425
175
176  CCG TGACACGGCG CCCACTTGCC ACCTAGGCGC TCTGGTGGCC CTTGCAGAAG      1531
177  Pro
178
179
180  CCCTAAGTAC GGTACTTAT GCGGTAGAC ATTTTATGTC ACTTATTAAG CCGCTGGCAC      1591
181
182  GGCCCTGCGT AGCAGCACCA GCCGGCCCCA CCCCTGCTCG CCCCTATCGC TCCAGCCAAG      1651
183
184  GCGAAGAAGC ACGAACGAAT GTCGAGAGGG GGTGAAGACA TTTCTCAACT TCTCGGCCGG      1711
185
186  AGTTTGGCTG AGATCGCGGT ATTAAATCTG TGAAAGAAAA CAAAACAAAA CAAAAAAAAA      1771
187
188  AAAAAAAAAA AA      1783
189
190
191  (2) INFORMATION FOR SEQ ID NO:2:
192
193      (i) SEQUENCE CHARACTERISTICS:
194          (A) LENGTH: 428 amino acids
195          (B) TYPE: amino acid
196          (D) TOPOLOGY: linear
197
198      (ii) MOLECULE TYPE: protein
199
200      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
201
202  Met Glu Glu Thr Gln Gln Gly Glu Ala Pro Arg Gly Gln Leu Arg Gly
203      1                      5                      10                      15
204
205  Glu Ser Ala Ala Pro Val Pro Gln Ala Leu Leu Leu Val Leu Leu Gly

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RAW SEQUENCE LISTING PATENT APPLICATION US/60/028,711

DATE: 11/29/96
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INPUT SET: S14126.raw

	20	25	30
206			
207			
208	Ala Arg Ala Gln Gly Gly Thr Arg Ser Pro Arg Cys Asp Cys Ala Gly		
209	35	40	45
210			
211	Asp Phe His Lys Lys Ile Gly Leu Phe Cys Cys Arg Gly Cys Pro Ala		
212	50	55	60
213			
214	Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro Cys Gly Asn Ser Thr		
215	65	70	75
216			
217	Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala Trp Glu Asn His His		
218	85	90	95
219			
220	Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp Glu Gln Ala Ser Gln		
221	100	105	110
222			
223	Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp Thr Arg Cys Gly Cys		
224	115	120	125
225			
226	Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser Gln Cys Val Ser Ser		
227	130	135	140
228			
229	Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys Gly Ala Leu His Arg		
230	145	150	155
231			
232	His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr Asp Cys Gly Thr Cys		
233	165	170	175
234			
235	Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys Val Ser Cys Pro Thr		
236	180	185	190
237			
238	Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala Ala Val Cys Gly Trp		
239	195	200	205
240			
241	Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala Gly Leu Val Val Pro		
242	210	215	220
243			
244	Leu Leu Leu Gly Ala Thr Leu Thr Tyr Thr Tyr Arg His Cys Trp Pro		
245	225	230	235
246			
247	His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly Met Glu Ala Leu Thr		
248	245	250	255
249			
250	Pro Pro Pro Ala Thr His Leu Ser Pro Leu Asp Ser Ala His Thr Leu		
251	260	265	270
252			
253	Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys Thr Val Gln Leu Val		
254	275	280	285
255			
256	Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr Gln Glu Ala Leu Cys		
257	290	295	300
258			

60/028711

Provisional Application For Patent Cover Sheet



Address:
Assistant Commissioner for Patents
Box Provisional Application
Washington, DC 20231

A request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(b)(2).

Docket Number: 1488.0310001

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this box ☒

+

INVENTOR(S)/APPLICANT(S)			
Last Name	First Name	Middle Initial	Residence (City and either State or Foreign Country)
1) YU	Guo-Liang		Darnestown, MD MD
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3) DIXIT	Vishva		Anarbor, MI MI
4) GENTZ	Reiner	L.	Silver Spring, MD
5) DILLON	Patrick	J.	Gaithersburg, MD MD
TITLE OF THE INVENTION (280 Characters Maximum)			
Death Domain Containing Receptors			
CORRESPONDENCE ADDRESS (including country if not United States)			
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Ave., NW, Suite 600			
State: <u>Washington, DC</u> Zip Code: <u>20005-3934</u> Country: <u>US</u>			
ENCLOSED APPLICATION PARTS (check all that apply)			
<input checked="" type="checkbox"/> Specification Number of pages: 92 <input type="checkbox"/> Small Entity Statement Including Sequence Listing and Claims <input checked="" type="checkbox"/> Drawing(s) Number of sheets: 6 <input checked="" type="checkbox"/> Other (specify) <u>Diskette copy of Sequence Listing</u>			
Method Of Payment (check one)			
<input checked="" type="checkbox"/> Our Check No. 17831 is enclosed to cover the Provisional filing fees. <input checked="" type="checkbox"/> The U.S. Patent and Trademark Office is hereby authorized to charge any deficiencies in the filing fees and credit any overpayments to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed. <input type="checkbox"/> The U.S. Patent and Trademark Office is hereby authorized to charge any fee associated with this filing to our Deposit Account No. 19-0036.		Provisional Filing Fee Amount (\$)	\$150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Signature: Kimberlin M. Toohy
 Typed or Printed Name: Kimberlin M. Toohy

Date: Oct 17, 1996
 Registration No. 35,391

Death Domain Containing Receptor

Abstract

The present invention relates to novel Death Domain Containing Receptor (DR3 and DR3-V1) proteins which are members of the tumor necrosis factor (TNF) receptor family. In particular, isolated nucleic acid molecules are provided encoding the human DR3 and DR3-V1 proteins. DR3 and DR3-V1 polypeptides are also provided as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR3 and DR3-V1 activity.

In Re Ashkenazi
Application No. 09/993,234
Amended Appeal Brief

10. RELATED PROCEEDINGS APPENDIX

None

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